THE UNIVERSITY
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THE DEVELOPMENT AND USE OF AN *IN VITRO* TECHNIQUE TO INVESTIGATE THE EFFECT OF PHARMACEUTICAL AGENTS ON FEMALE GERM CELL DEVELOPMENT

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and work of all others is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

AstraZeneca approved the data presented in this thesis and some chemical compound information is not given due to the confidentiality agreement with AstraZeneca.

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Abstract

With meiosis spanning from embryonic development to the end of reproductive life in females, scientists have faced considerable limitations in studying female meiosis and the effects of toxicants on the developing oocyte. Over the last half century, various culture methods have been developed with the aim of studying the mechanisms of early ovary development, as well as for use in reproductive toxicology. However, very few of the established embryonic ovary culture systems have been used to investigate potential reproductive toxicants on the embryonic ovary, in particular when compared with the vast number of *in vitro* reproductive toxicity studies on the post-natal ovary. Here, a novel test compound, a topoisomerase II inhibitor: AstraZeneca Test Compound (AZTC), was used to investigate the efficacy and validity of ovarian culture methods when compared with *in vivo* reprotoxicity studies. AZTC was selected due to preliminary *in vivo* studies demonstrating its detrimental effects on spermatogenesis in male rats. AZTC targets bacterial type II topoisomerases that might have mammalian homologues involved in meiosis.

Topoisomerase-II α was expressed within the female germ cells pre-natally, but became localised to the granulosa and stroma cells post-natally. This occurred both *in vivo* and *in vitro*. Ovaries from female rats exposed pre-natally to AZTC *in vivo* were analysed histologically and a significant increase in the number of primordial follicles was observed within the ovaries, as well as an increase in the number of unhealthy follicles.

A novel mouse embryonic ovary culture system was developed by adapting, improving and bridging existing available culture techniques. The culture system supported growth of pre-meiotic mouse germ cells through prophase I of meiosis, the formation of primordial follicles and initiation of follicle growth. Cultured ovaries contained follicles at stages in comparable ratios to those *in vivo* and appeared

morphologically normal and healthy. The culture also supported meiotic progression of oocytes to the pachytene stage, albeit with a slight delay.

AZTC was used to validate the novel embryonic ovary culture by comparing the results with those from the *in vivo* study, where AZTC exposure had also occurred during embryonic development. Similar results were consistently observed between the *in vivo* and *in vitro* studies. *In vitro* effects of AZTC on the post-natal mouse ovary were also investigated, where neonatal mouse ovaries cultured with AZTC had fewer primordial follicles and more unhealthy follicles than did control ovaries. AZTC therefore demonstrated different effects when exposure occurred pre-natally vs. post-natally. The embryonic ovary culture was then used to examine the effects of another topoisomerase II inhibitor, etoposide, on the pre-natal ovary. Etoposide is a chemotherapy agent and has previously been prescribed to pregnant women. A significant reduction in the size of the follicle pool was observed in exposed cultured embryonic ovaries, where primordial and transitional follicles were targeted.

Overall, establishment of post-natal culture systems have become a useful addition to *in vivo* reproductive toxicology studies. The embryonic ovary culture system developed here could become a valuable and powerful tool to screen potential reproductive toxicants, as well as to study the dynamics and regulation of early ovary development.

Publication arising from this work

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Abstracts for poster presentations

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'The effect on female fertility of conditionally deleting Foxg1 in the ovary'

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Agnes Stefansdottir, Richard Anderson, Ian Adams, Nicola Powles-Glover, Norah Spears

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Assistance given throughout this investigation

All work detailed throughout this thesis was conducted by myself with the following exceptions:

- **Chapter 3**: The initial work regarding the AZTC *in vivo* study, including dosing, ovary collection, fixation and embedding was carried out by AstraZeneca staff. I was helped in carrying out the *in vivo* mouse Topo IIα immunostaining by Kevin Randall at Alderley Park (AstraZeneca).

- **Chapter 4**: I was helped by Ian Adams, Diana Best and Judith Reichmann in setting up the initial early embryonic ovary culture.

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Abbreviations

AKT	Protein Kinase B
ATP	Adenosine Triphosphate
AMH	Anti-Mullerian Hormone
Apaf	Apoptotic protease activating factor 1
AZTC	AstraZeneca Test Compound
BAX	Bcl-2-associated X protein
BAD	Bcl-2-associated death promoter
B[a]P	Benzo(a)pyrene
BCL2	B-cell lymphoma 2
BID	BH3 Interacting-domain Death Agonist
BM	Basement membrane
BMP	Bone Morphogenetic Protein
BPA	Bisphenol A
BSA	Bovine serum albumin
CL	Corpus Luteum
CARD	Caspase recruitment domains
COC	The cumulus oocyte complex
DAB	3,3'-Diaminobenzidine
DAZL	Deleted In Azoospermia-Like
DD	Death domains
DES	Diethylstylbestrol
DMBA	Dimethyl(a)anthracene
DMC1	Disrupted meiotic cDNA1
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic Acid
DSB	Double-strand breaks
DXR	Doxorubicin
E1	Estrone
E2	Estradiol
EDC	Endocrine disrupting compounds
ER	Estrogen Receptor
FAS	FAS receptor/Apoptosis antigen 1
FIGLA	Folliculogenesis specific basic helix-loop-helix
FOXL2	Forkhead Box L2
FOXO3A	Forkhead Box O3
FSH	Follicle Stimulating Hormone
GDF9	Growth Differentiation Factor-9
GnRH	Gonadotrophin-releasing hormone
HA	Hyaluronan
HSP90	The 90kD family of heat shock proteins
H&E	Hematoxylin and eosin
IGF1	Insulin-like Growth Factor 1
KL	Kit Ligand
L-15	Liebovitz
LH	Luteneizing hormone
mRNA	Messenger RNA
MOF	Multioocyte follicle
NGF	Nerve Growth Factor
NGS	Normal goat serum

NOBOX	Newborn Ovary Homeobox Protein
NOXA	Phorbol-12-myristate-13-acetate-induced protein
P27	Cyclin Dependent Kinase Inhibitor 1B
P53	Tumour suppressor protein p53
PAH	Polyclic Aromatic Hydrocarbon
PBS	Phosphate buffered saline
PCD	Programmed cell death
PGCs	Primordial germ cells
PgE2	Prostaglandin E2
PGS-2	Prostaglandin Endoperoxide Synthase-2
PI3K	Phosphoinositide 3-kinase
PND	Post-natal day
POI	Premature ovarian insufficiency
PP	Post partum
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
PUMA	P53 regulated modulator of apoptosis
RA	Retinoic Acid
RAD51	RAD51 Recombinase
REC8	Meiotic Recombination Protein
RNA	Ribonucleic Acid
RPSO	RNA Polymerase, Sigma S
SC	Synaptonemal Complex
SCF	Stem Cell Factor
SDF1	Stromal Cell-derived Factor 1

SMC1b/3	Structural Maintenance of Chromosomes Protein 1b/3
STAG3	Stromal Antigen
STRA8	Stimulated by Retinoic Acid 8
SYCE	Synaptonemal Complex Central Element Protein
SYCP	Synaptonemal Complex Protein
TEX12	Testis Expressed 12
TF	Transcription Factor
TGFβ	Transforming Growth Factor β
TNF	Tumor Necrosis Factor
Topo II	Topoisomerase II
TZPs	Tranzonal projections
USEPA	United States Environmental Protection Agency
ZAR1	Zygote Arrest 1
ZP	Zona Pellucida

Chapter 1.
General Introduction

1. Introduction

Female germ cells, or oocytes, undergo an extended process of meiosis, which begins before birth and in some cases, is not completed until the end of the female's reproductive lifespan. Oocytes become arrested in prophase I of meiosis before becoming enclosed in primordial follicles so that by birth, the ovary contains a pool of resting primordial follicles. This primordial follicle pool plays a crucial role in determining the future fertility of the female, as it is from this pool of follicles that all future oocytes will be ovulated. The pool will become gradually depleted as oocytes exit the resting pool and begin to grow and develop toward the pre-ovulatory stage. Meiosis is not resumed until the oocyte is ovulated and this stop-and-start process of meiosis makes assessment of any external compounds on the ovary difficult, yet that much more crucial. If the primordial follicle pool is affected, this can result in premature ovarian ageing and potentially premature menopause. However, many of the factors and processes involved in the regulation of early oocyte development and follicle formation are not fully understood.

The rodent ovary is an excellent model for *in vitro* studies, partly due to its relatively short time-course. Various culture techniques in mice have been developed that span various stages of ovary and follicle development, including an embryonic ovary culture system and a newborn ovary culture system. Combining these two culture systems to generate a culture system that would span from meiotic entry of oogonia, through germ cell nest breakdown, follicle formation and initiation of follicle growth, would not only be a valuable tool in the study of the dynamics and regulation of ovary development, but also a useful model to screen potential toxicants on the pre-natal ovary.

Current reproductive toxicity testing of pharmaceuticals on the pre-natal ovary involves assessing the reproductive performance of females that have been exposed in utero. Although this study design will detect any immediate reproductive problems, it does not detect any subtle effects the compound may have on the primordial follicle pool which could potentially affect long term fertility. For example, there are a number of antibiotics that act by targeting bacterial

topoisomerases. Many of these will have mammalian homologues involved in meiosis, and could therefore affect spermatogenesis or early ovary development, potentially resulting in an irrevocably diminished pool of oocytes.

The aim of this PhD was therefore to bridge, adapt and improve existing culture methods available in Edinburgh, namely, the embryonic and neonatal ovary culture systems, to generate a culture system spanning early ovary development, from meiotic entry through to follicle formation and growth. The second aim of the PhD was to investigate the potential effects of a novel pharmaceutical test agent, a topoisomerase II inhibitor: 'AstraZeneca Test Compound' (AZTC) provided by AstraZeneca, on the embryonic ovary culture system.

1.1. The ovary

The ovary is central to female reproductive function, the site within which germ cells form follicles; functional unit of the ovary. Germ cells are responsible for carrying genetic material from one generation to the next, and they do this by generating haploid cells via a unique type of cell division: meiosis. In female mammals, oocytes develop from primordial germ cells (PGCs) during gestation (Baillet and Mandon-Pepin, 2012). Following the proliferative stage of primordial germ cells, the PGCs enter a pre-meiotic state of DNA replication before entering prophase I of meiosis. They then progress through the initial stages of meiosis, before undergoing a state of meiotic arrest, around the time of follicle formation (Anderson and Hirshfield, 1992, Adams and McLaren, 2002). The oocytes remain in this meiotically arrested state throughout the phase of follicular development. In the post-natal ovary, as an oocyte grows and matures, its follicle undergoes changes due to proliferation of the granulosa cells and formation of the fluid filled antral cavity, resulting in a dramatic increase in follicle size. Once the pre-ovulatory, Graafian follicle has reached full maturation, it expels its oocyte during ovulation, at which point the oocyte exits meiotic arrest and completes meiosis I.

The ovary is not only responsible for producing oocytes, but is also an important endocrine gland, the source of sex steroids which link reproductive and non-reproductive organs to the timing of the ovarian cycle. It is in the growing follicle that the majority of estrogens in the body are produced and once the oocyte has been ovulated, the remainder of the follicle becomes a corpus luteum (CL), a temporary endocrine structure secreting the progesterone critical for the establishment and initial maintenance of pregnancy. The ovary is responsive to hormones secreted from the anterior pituitary, in turn controlled by the hypothalamus, with which it is locked into a complex cyclical pattern of communication and feedback that underpins successful female reproduction.

There is a certain amount of disparity involving folliculogenesis, follicle growth and other reproductive factors between different species. Research using animal models for investigating human ovarian function must therefore be carried out with care, providing a hypothetical basis for study. Three commonly used animal models used in reproductive biology are the mouse, sheep and bovine models, with each model differing slightly from the human in different ways. For example, while the mouse, bovine and human all undergo menstrual/estrous cycles all year around, the ewe is a seasonal breeder. Follicle formation also occurs at slightly different time-points depending on the species. In human, ewe and bovine ovaries, follicles first begin to form during pre-natal ovary development. This occurs around weeks 16-20 of human gestation, around days 70-90 of bovine gestation and around day 170 of bovine gestation (Hartshorne et al., 2009, Aerts and Bols, 2010, McNatty et al., 1995, Smith et al., 2014). In the mouse ovary on the other hand, the first follicles do not form until around birth (Sarraj and Drummond, 2012) . Furthermore, while the bovine and ewe are mono-ovular, like the human, the mouse model is poly-ovular. Although a large majority of available information on the ovary and female reproduction comes from studies carried out on these three animal models, this thesis will concentrate on the mouse model because all the culture work presented in this thesis was carried out on the mouse model. From this point onwards I will therefore refer to the mouse model, unless otherwise indicated.

1.1.1. Pre-natal ovary development

1.1.1.1. The formation and migration of female germ cells

The formation of female gametes, oocytes, begins in the developing fetus with the formation of PGCs. Pluripotent cells in the epiblast of the developing mouse embryo differentiate into PGCs around embryonic day 6.5 (E6.5), with E0.5 being the morning a vaginal plug is found, approximately half a day after conception (Ohinata et al., 2005). The PGCs enter a proliferative phase, during which they migrate to the developing genital ridges which will later become the ovaries in female embryos. It has been suggested that PGC migration occurs in response to an attractant, such as stromal cell-derived factor 1 (SDF1) or kit ligand (KL), also known as stem cell factor (SCF), originating in the genital ridges (Farini et al., 2007). The PGCs invade the developing ovary around embryonic day 10.5 (E10.5), or week 7 of human gestation. From this point onwards they become known as oogonia (Anderson and Hirshfield, 1992). Once in the ovary, the oogonia continue to proliferate, forming germ cell nests or germline cysts due to a series of incomplete cell divisions, resulting in the formation of clusters of connected cells (Pepling and Spradling, 1998). The proliferating oogonia then begin to receive signals from the somatic cells of the ovary, which subsequently makes their development become more directed (Hajkova et al., 2008, Hajkova et al., 2002). The oogonia stop proliferating and enter a pre-meiotic state around E12.5, or between the 8th and the 13th week of human gestation (Oktem and Urman, 2010).

1.1.1.2. Female Meiosis

Meiosis is a central event in the generation of germ cells of both sexes. It reduces the complement of chromosomes to form haploid gametes containing only one copy of each chromosome. However, while meiotic progression in male gametes is continuous, with spermatogenesis occurring at a constant rate, in females of most species (including humans and rodents), it contains two constitutive arrest phases.

Meiotic initiation is asynchronous in both rodent and human ovaries, and variation in meiotic stage is not uncommon between neighbouring oocytes of the mouse ovary (Gondos, 1987, Hartshorne et al., 2009). Meiosis begins in the developing ovary of

the female mouse embryo around E13.5, following the proliferative phase of oogonia. From this point onwards, the germ cells are termed oocytes. In human fetal development, germ cells enter meiosis in the 3rd month of gestation, but in contrast to the mouse ovary which has a smaller window of meiotic entry, some human germ cells continue to proliferate for a long time after the first germ cells enter meiosis. There is, therefore, a long a time-frame for oocytes entering meiosis in the human ovary, whereas the time-span for meiotic entry in the mouse is much smaller (Baker, 1963, Bendsen et al., 2006, Fulton et al., 2005, Anderson et al., 2007, Childs et al., 2012).

Prior to meiotic entry, primordial germ cells enter a transient pre-meiotic state of DNA replication, the S-phase of interphase, before entering prophase I of meiosis (Anderson and Hirshfield, 1992). Prophase I of meiosis consists of five stages: leptotene (chromosome condensation), zygotene (beginning of synapsis), pachytene (complete synapsis and crossover), diplotene (separation of homologous chromosomes, remaining attached at chiasmata) and diakinesis (separation of chromosomes, except for at the terminal chiasmata) (Fig. 1.1). Oocytes arrest at the diplotene stage and remain in this meiotically arrested state until ovulation. They are therefore in this dormant state of meiotic arrest throughout follicular development.

In rodents, the oogonia that are closest to the mesonephros are first to enter meiosis, possibly due to the migration of small diffusible molecule, retinoic acid (RA), that originates in the ovary and the mesonephros (Mu et al., 2012, Bowles and Koopman, 2007a). RA is thought to be one of the critical meiosis-inducing factors in the developing ovary, along with the RA-inducible STRA8 protein, and DAZL, which is an RNA-binding protein expressed in the germ cells (Hartshorne et al., 2009, Lin et al., 2008, Baltus et al., 2006, Bowles and Koopman, 2007b). Although initially, mesonephros derived RA was believed to be required for initiating germ cell meiosis in the fetal ovary, a recent study has demonstrated how ovary derived RA alone is responsible for meiosis initiation (Mu et al., 2013). Various other factors have also been identified as being involved in prophase I of meiosis. During the pre-meiotic phase, cohesin proteins (REC8, SMC1b, SMC3 and STAG3) are required for the

attachment of the new sister chromatid to the older chromatid (Baillet and Mandon-Pepin, 2012). During leptotene, the synaptonemal complex (SC), consisting of a transversal element (SYCP1), an axial element (SYCP2 and SYCP3) and a central element (SYCE1, SYCE2 and TEX12), is required for pairing of homologous chromosomes (Baillet and Mandon-Pepin, 2012). Recombinase proteins DMX1 and RAD51 are required to interact with SYCP3 to induce synapsis of chromosomes (Tarsounas et al., 1999). The leptotene stage is also associated with double-strand breaks (DSBs), which are required for genetic recombination. DSBs are triggered by SPO11 (Romanienko and Camerini-Otero, 2000), and the TEX15 protein assists in the localisation of DMC1 and RAD51 to the DSB (Yang et al., 2008a). During zygotene, the formation of the SC is dependent on the interaction between TEX11 and SYCP2 (Yang et al., 2008b). Once the SC is fully formed at pachytene, crossovers are initiated (SC formation is further outlined in Section 4.13). Analysis of SYCP3 protein expression is found in E12.5 mouse ovaries, but it appears in over 80% of female germ cells by E13.5. Mouse germ cells are therefore in pre-meiotic stage at E12.5 and enter prophase I of meiosis at E13.5 (Shen et al., 2006).

Throughout follicular development, the oocyte slowly gains the competency for nuclear and cytoplasmic maturation, but it is only following the luteneizing hormone (LH) surge of ovulation that the oocyte goes on to complete the diakinesis stage of prophase I (Fig. 1.1) and completes meiosis I. The oocyte then enters the second meiotic division and arrests again at metaphase II where it remains arrested until fertilization. In contrast to meiosis in spermatogonia, each female meiotic division results in the formation of one daughter cell (the oocyte) and two polar bodies. Due to an uneven distribution of cytoplasm, the daughter cell receives the majority of the cytoplasm, with the polar bodies containing little material other than the expelled chromosomes: these polar bodies later disintegrate.

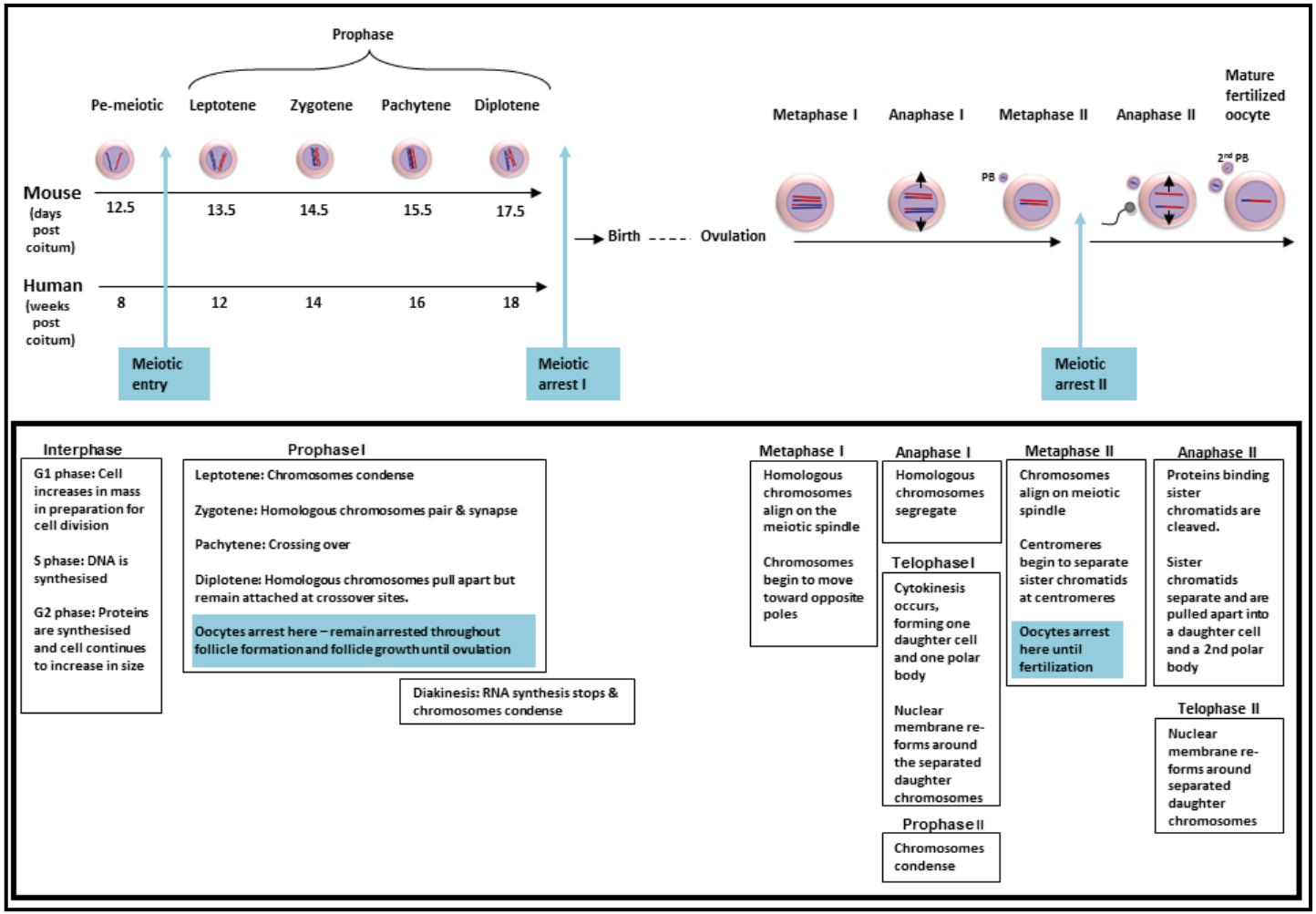


Figure 1.1. Meiosis in oocytes

Stages and phases of mammalian meiosis, including two meiotic arrests during Prophase I and Metaphase II. The figure demonstrates chromosomal behaviour during meiotic progression in the mouse and human, with the time-course of events leading to the formation of an arrested oocyte within the growing follicle and ultimately, a mature fertilized oocyte.

1.1.1.3. Germ cell nest breakdown

The creation of the primordial follicle pool is a tightly regulated and multi-faceted developmental process. Pre-natal oogenesis of the human ovary results, initially, in the production of around 7 million oogonia, but by birth, these have reduced from 7 million to between 2 million to 70,000 due to pre-natal germ cell loss (Oktem and Urman, 2010).

Oogonia that have invaded the developing ovary are initially arranged in germ cell nests, which form due to a series of incomplete synchronous mitotic divisions of the oogonia. As they divide, cytokinesis is not completed and therefore the daughter cells are all connected by intercellular bridges. Germ cell nests form between meiotic entry and follicle formation the human ovary around the 9th week of gestation and between E10.5-13.5 in the mouse ovary, just prior to their entry into the meiotic prophase (Pepling and Spradling, 2001). It has also been suggested that germ cell nests can form due to cell aggregation (Bendel-Stenzel et al., 2000, Mork et al., 2012).

Germ cell nest breakdown is associated with an estimated loss of around one-third to two-thirds of oogonia (Pepling and Spradling, 2001, Bristol-Gould et al., 2006). The purpose of this germ cell loss is not well understood, but it has been proposed to be a way to eliminate germ cells with chromosomal abnormalities or less functional mitochondrial genotypes (Pepling and Spradling, 2001). In the mouse, mitochondria enter a rapid phase of division right before germ cell nest breakdown, and mitochondria have been detected within the intracellular bridges between oogonia (Pepling and Spradling, 2001). This supports the possibility that nurse cells exist only to produce and transfer nutrients and organelles to the oocyte once germ cell breakdown occurs. Another possibility is that certain germ cells within the nest become nurse cells and take on a supporting role to assist the germ cells that will later go on to become oocytes (de Cuevas et al., 1997). It is still unknown what factors control which oocytes are selected for survival or to undergo apoptosis, but estrogen has been suggested to play a role in this process (Zachos et al., 2002).

Steroid hormones, such as estradiol (E2), have been implicated in the regulation of cell death in various tissues (Kyprianou et al., 1991, Perillo et al., 2000, Sawada and Shimohama, 2000). In the developing ovary, E2 inhibits germ cell nest breakdown and protects oocytes and granulosa cells from programmed cell death (Billig et al., 1993). High estrogen exposure interferes with germ cell nest breakdown and can, in some cases result in multioocyte follicles (MOFs). MOFs are follicles containing two or more oocytes within a single follicle, with no basement membrane separating them (Tingen et al., 2009). Exposure to estrogenic compounds during the time of germ cell nest breakdown has been linked to MOF formation in adult mouse ovaries (Iguchi et al., 1990, Cimafranca et al., 2010, Jefferson et al., 2007). Oocytes in MOFs have a 30% lower fertilization rate than mono-ovular oocytes (Iguchi et al., 1991), demonstrating the importance of germ cell nest breakdown for oocyte quality (Tingen et al., 2009).

1.1.1.4. Follicle formation

As germ cell nests break down, isolated oocytes, which at this point have entered meiotic arrest, go on to form primordial follicles. A primordial follicle consists of the arrested oocyte (about 15-20 μm in diameter in rodents, and around 30 μm in humans) surrounded by flattened somatic cells, called pre-granulosa cell (Moniruzzaman and Miyano, 2010). The period of germ cell nest breakdown and formation of primordial follicles is a crucial one, as the primordial follicles represent the pool of follicles from which all future oocytes will be ovulated. By the time primordial follicles have formed oocyte numbers have already vastly decreased. From this point onwards, oocyte numbers continue to decrease as the vast majority of primordial follicles that enter the growing pool of follicles undergo atresia and die (Oktem and Urman, 2010). Primordial follicles usually end up occupying the cortex of the ovary, with growing follicles located more centrally.

Several genes are critical for early differentiation of the ovary, many of which are members of the β-catenin pathway. These include both extracellular paracrine or autocrine factors such as RPSO and WNT4, as well as intracellular transcription factors (TF) such as FOXL2 (Crisponi et al., 2001, Parma et al., 2006, Chassot et al.,

2008, Pailhoux et al., 2001, Liu et al., 2009a). Furthermore, numerous oocyte-specific genes have been identified whose transcription is important for initiating follicle formation. These include TFs *Gdf9*, *Zar1, Ngf, Figla* and *nobox,* which are all expressed within oocytes of germ cell clusters and/or primordial follicles (McGrath et al., 1995).

The primordial follicle pool is believed to be non-renewable. That is, once all germ cells have entered meiosis, with no mitotic oogonia left, then no further oocytes can form. However, some recent research has suggested the existence of germline stem cells within the ovary, proposing that they are used to replenish the primordial follicle pool (Johnson et al., 2004, Zou et al., 2009, White et al., 2012). Their location within the ovary is still unknown however, and more studies are needed before we can be certain of their existence.

1.1.2. Post-natal ovary development

Primordial follicles become recruited to grow pre-natally in the human ovary, and around or shortly after birth in the mouse. Follicle recruitment continues throughout life for most species, whereas in the human ovary it carries on until the primordial follicle pool has been depleted, at which point the woman reaches menopause. The size of the primordial follicle pool is therefore a key determinant of a woman's reproductive life span (Oktem and Urman, 2010).

1.1.2.1. Initiation of follicle growth

The first step in the growth of the follicle occurs when a follicle from the resting pool of primordial follicles, surrounded by flattened granulosa cells, become stimulated to grow. Once activated, the granulosa cells of primordial follicles become cuboidal and the follicle becomes a primary follicle. Secondary follicles are formed as granulosa cells begin to proliferate and the follicle becomes multi-layered (Fig.1.2).

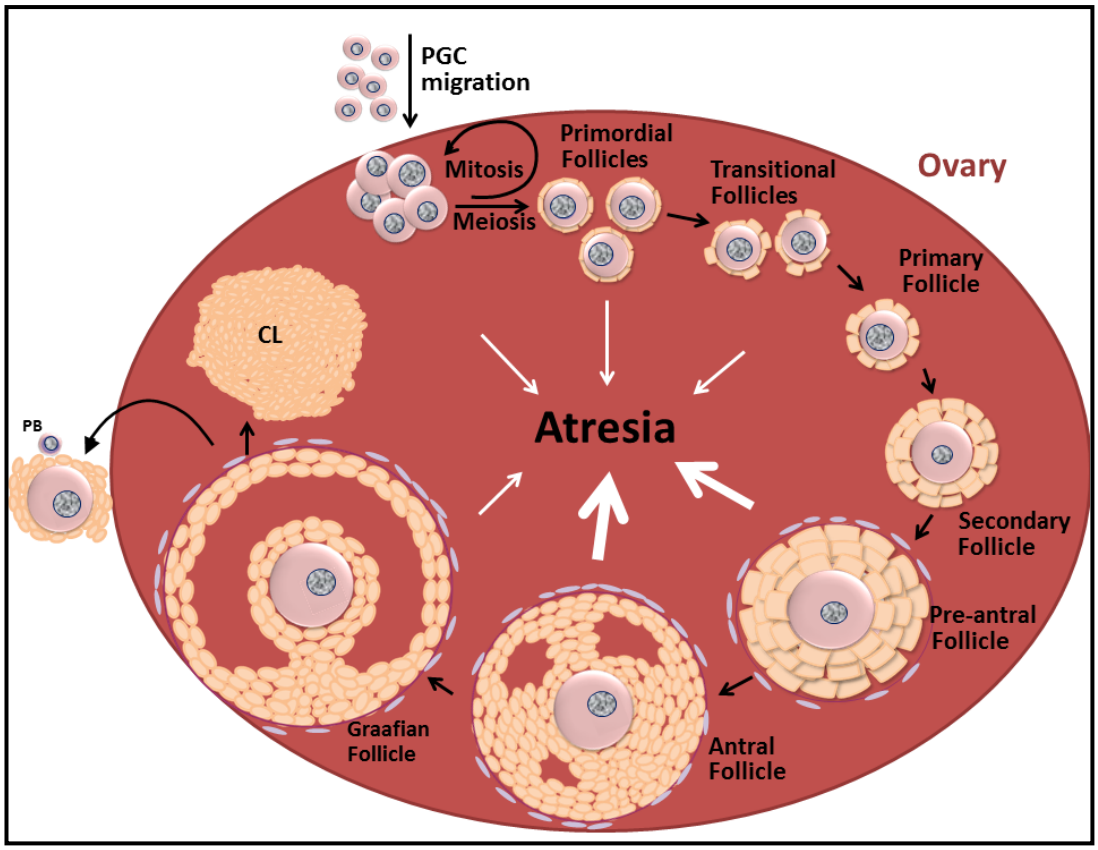


Figure 1.2. Follicle growth in a human ovary.

Proliferating PGCs migrate to, and invade the developing ovary to form germ cell nests. They proliferate at a high rate, then enter meiosis and form primordial follicles as the germ cell nests break down. Throughout the reproductive lifespan, small cohorts of primordial follicles are continually released from dormancy as they enter the growing pool. The vast majority of growing follicles are lost to atresia, but beginning at puberty, a few follicles grow to the Graafian stage, normally resulting in the release of one oocyte each menstrual cycle. The remainder of the ovulatory follicle forms the CL.

Early stages of follicle growth occur independently of gonadotropins. They are instead reliant on local factors derived from both somatic (granulosa and theca) and germ cells creating an orchestrated multi-directional signalling pathway that drives the primordial-to-primary follicle transition. The various factors involved in the regulation of follicle recruitment include inhibitory factors as well as stimulatory ones (Fig 1.3).

Inhibitory factors are important for maintaining primordial follicles in their dormant state. Knock-out mouse models of genes involved in maintaining follicular quiescence (such as *Pten, p27, Foxl2* and *Foxo3a*) all result in wholescale premature follicle activation and subsequently, early exhaustion of the primordial follicle pool (Castrillion et al., 2003, Adhikari et al., 2010). Foxo3a, a member of the FOXO subfamily of forkhead TFs, is one of the key regulators of early follicle growth, a downstream effector of the PTEN/PI3K/AKT pathway (Liu et al., 2009b). Females deficient in Foxo3a exhibit accelerated follicular activation and secondary infertility (Castrillion et al., 2003). Furthermore, mutations in the *Foxl2* gene in the human ovary have been linked to premature ovarian insufficiency, emphasising the importance of inhibitory factors for female fertility (De Baere et al., 2003). Anti-mullerian hormone (AMH), another member of the TGFβ family, has been implicated in inhibiting primordial follicle activation. AMH is expressed in granulosa cells of growing follicles and, when inhibited, an increase in primordial-to-primary transitions is observed (McGee and Hsueh, 2000, Durlinger et al., 2002, Durlinger et al., 1999).

Several members of the TGFβ family, such as BMP-4, BMP-7 and GDF-9, play crucial roles in primordial follicle activation. GDF-9 is an oocyte derived factor whereas BMP-4 and BMP-7 are expressed in theca and stromal cells (Lee et al., 2001, Nilsson and Skinner, 2003, Carbatos et al., 1998, Vitt et al., 2000). Other growth factors implicated include KL and LIF, which stimulate oocyte growth and theca cell recruitment (Nilsson and Skinner, 2004). The growth factors involved in the oocyte-granulosa cell regulatory loop are described in further detail in the next section (1.1.2.2).

1.1.2.2 Oocyte-somatic cell interactions (gap junctions, tranzonal projections)

The ovarian follicle is a highly functional unit in which somatic and germ cells are closely associated and co-dependent. The follicle requires appropriate oocyte-somatic cell interactions for growth. Oocytes drive follicle development by secreting TGFβ superfamily members GDF9 and BMP15. These growth factors regulate proliferation of granulosa cells in primary follicles as well as promoting theca cell differentiation (McGee and Hsueh, 2000, Orisaka et al., 2009). Initiation of primordial follicle growth to the primary follicle stage is un-affected in mice with mutations in the *Gdf9* gene (Dong et al., 1996). However, follicles in these ovaries are unable to form secondary follicles, characterized by a failure in the theca cell layer formation (Elvin et al., 1999). Furthermore GDF9 augments androgen production in pre-antral follicles. GDF9 is therefore thought to be crucial for pre-antral follicle growth beyond the primary follicle stage where it might stimulate the recruitment, proliferation and differentiation of theca cells (Orisaka et al., 2009). GDF9 also suppresses KL expression in granulosa cells of antral follicles (Joyce et al., 1999, Joyce et al., 2000). In turn, KL is thought to promote oocyte growth and development. It is expressed in the granulosa cells and its receptor, c-kit, is expressed within the oocyte and theca cells, which further suggests a strong functional relationship between oocytes and granulosa cells (Nilsson and Skinner, 2004, Nilsson et al., 2002). However, there is some debate over the role of KL in early follicle development. One study (Parrott and Skinner, 1999) suggested that KL induces follicle development, whereas a different study suggested that it is involved in promoting oocyte survival rather than follicle activation directly, as following KL exposure they found no increase in the number of growing follicles, but instead reported a higher number of surviving primordial oocytes within the ovaries (Moniruzzaman and Miyano, 2010). FSH has also been shown to promote KL expression in granulosa cells of pre-antral follicles, but not in antral follicles (Joyce et al., 1999). BMP15 is thought to play a similar functional role in early follicular development as GDF9, where synergism between GDF9 and BMP15 might be required for follicle development, although this varies somewhat between species (Eppig, 2001). GDF9 and BMP15 promote granulosa cell proliferation in small antral follicles (Hayashi et al., 1999, Vitt et al., 2000, Otsuka et al., 2000), demonstrating

the importance of oocyte derived factors to drive granulosa cell proliferation. It is clear that the oocyte-granulosa-theca cell regulatory loop is a very complex one, with the oocyte playing a very active role in driving follicle growth, and the granulosa and theca cells secreting factors that in turn, regulate oocyte development.

Gap junctions are responsible for mediating these oocyte-granulosa cell and granulosa-granulosa cell signals, linking the granulosa cells to each other as well as to the oocyte (Anderson and Albertini, 1976). Gap junctions between oocytes and somatic cells start to form during fetal life and become established in primordial follicles of the neonatal mouse ovary (de Felici et al., 1989, Mitchell and Burghardt, 1986).

Trans-zonal projections (TZPs) are cytoplasmic processes that form within the granulosa cells, penetrate through the ZP and terminate at the oocyte plasma membrane (Albertini et al., 2001). TZP co-ordinate the bi-directional paracrine communication between oocytes and granulosa cells. Their numbers vary according to follicle stage, where they are most numerous during peak periods of oocyte growth but retracting during ovulation (Motta et al., 1994).

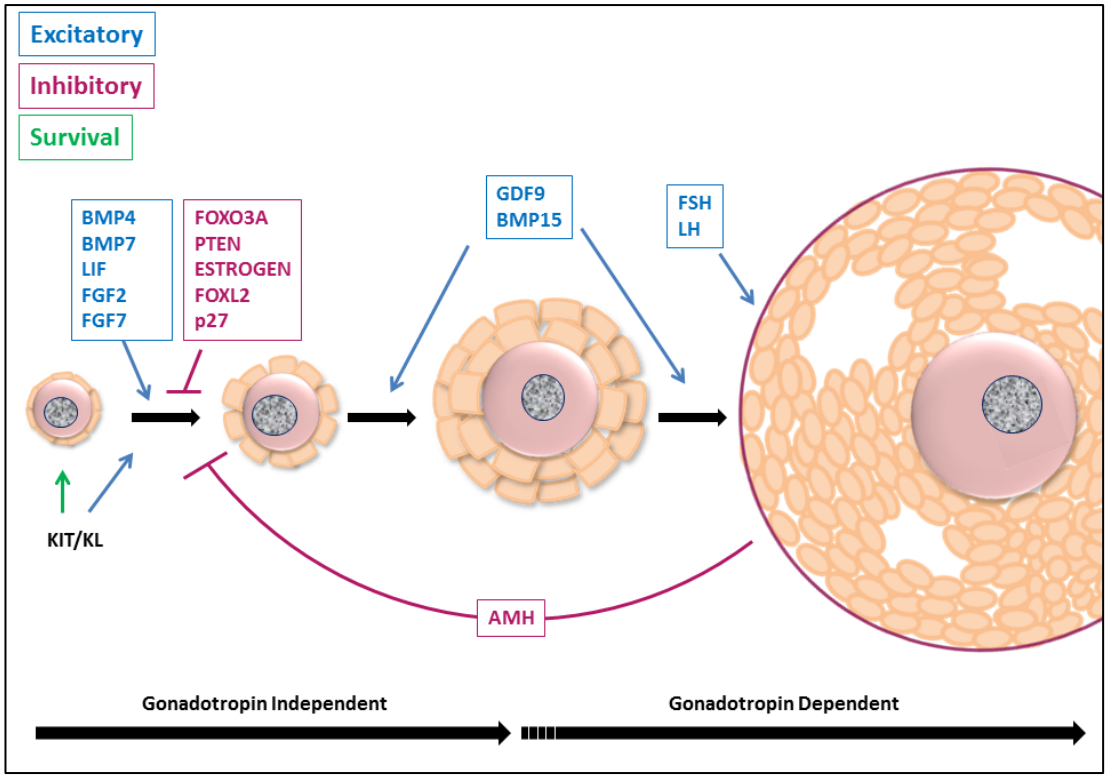


Figure 1.3. The main factors involved in the dynamics of follicle development. Growth initiation of primordial follicles and subsequent development of the follicle through the primary, secondary and tertiary stages is finely regulated by several inhibitory and excitatory growth factors and/or hormones. Growth of the follicle to the secondary stage is gonadotropin independent, whereas further growth to the antral stages of follicle development are gonadotropin responsive, requiring the presence of gonadotropins such as LH and FSH, in order to form a pre-ovulatory, Graafian follicle.

1.1.2.3. Follicle growth to the antral stage

As follicles progress through the primary, secondary and early antral stages of development, successive layers of granulosa cells form around the oocyte. Antral follicles eventually form when the proliferating granulosa cells secrete a viscous fluid that builds up, forming a fluid filled antrum. This stage is associated with an increase in follicle size and in oocyte diameter, as well as the establishment of basal lamina, zona pellucida (ZP) and a theca layer (Knight and Glister, 2006).

Once a follicle transits into the secondary, pre-antral stage, its growth onwards to the antral stage becomes gonadotropin responsive, with further growth dependent on gonadotropins such as follicle stimulating hormone (FSH) (Craig et al., 2007). Although FSH receptors are expressed in follicles prior to this, during pre-antral follicle development, FSH does not play an essential role in follicle growth until follicles have reached the antral stage of development (Oktay et al., 1997). At this point, FSH and LH receptors are further up-regulated and further follicle growth becomes dependent on gondadotropins.

1.1.2.4. Ovulation

Ovulation is a complex and highly specialised process that results in the release of a mature, fertilizable oocyte from a pre-ovulatory, Graafian follicle. Normally one oocyte is released per ovulatory cycle in humans, whereas litter-bearing animals will release more. The whole process involves many different cell types within the ovary, including the mural granulosa, cumulus and theca cells of the follicle, as well as stromal cells and the cells of the ovarian surface epithelium (Richards et al., 1998). Successful ovulation is reliant on efficient communication between the oocyte and its surrounding cells, as the follicle microenvironment is crucial for the culminating stages of oocyte differentiation, its nuclear and cytoplasmic growth and capacitation (Hennet and Combelles, 2012). Furthermore, the basal lamina and antral fluid also have a role to play in oocyte differentiation. Antral fluid is a rich fluid derived from the bloodstream and from components secreted by the granulosa cells. It contains a wide range of molecules such as steroid hormones, electrolytes and enzymes. It plays an important role in mediating signals between the different cells types of the follicle (Hennet and Combelles, 2012).

The hypothalamus, pituitary and ovary are the three main sites of sex hormone production in the body. They form a complex, intricate feedback loop to control the menstrual cycle in women. The human menstrual cycle can be divided into three parts: 1) The follicular phase, during which secondary follicles develop to the Graafian stage, 2) Ovulation, when a follicle has reached full maturation and an oocyte is released from the ovary and finally 3) the luteal, or post-ovulatory, phase which occurs following ovulation when the ruptured follicle transforms into the CL.

During the follicular phase of the ovulatory cycle, the hypothalamus releases pulses of gonadotrophin-releasing hormone (GnRH), which stimulates synthesis and secretion of two gonadotrophins, LH and FSH from the anterior pituitary. LH binds to receptors in theca cells and drives the conversion of cholesterol to androgen, which in turn is converted to oestrogens E2 and oestrone (E1) under the influence of aromatase in the granulosa cells. The FSH signal drives this conversion of theca-derived androgens to oestrogens in the granulosa cell (Fig. 1.4) (Craig et al., 2007). The increase in progesterone released from the granulosa cells activates the A-kinase pathway, inducing the expression of genes needed for ovulation, such as prostaglandin endoperoxide synthase-2 (PGS-2) and the progesterone receptor (PR) (Park and Mayo, 1991, Espey, 1980, Richards, 1994). The increase in progesterone levels also activates the production of prostaglandin E1 (PgE1), a vasoactive substance that causes a dilation of capillaries in the theca layers, resulting in a degradation of the extracellular matrix of the collagenous connective tissue (Hoyer, 2010). Rising FSH levels allow the dominant follicle to secrete increasing amounts of E2, inhibin and activin, hormones that negatively regulate the synthesis and release of FSH from the pituitary, inhibiting the remaining growing follicles which become atretic. The rising estrogen production exerts a negative feedback on the GnRH production in the hypothalamus. Oestrogen production then peaks just before ovulation and the granulosa cells of the dominant follicle also increase their secretion of inhibin and progesterone (Fortune, 1994). At this point, the negative feedback effect of oestrogen on GnRH earlier in the cycle changes to a positive feedback and as a result, a surge of LH secretion follows, driving ovulation.

The cumulus oocyte complex (COC) of the Graafian follicle is made up of the pre-ovulatory oocyte and the cumulus cells that surround it. The COC is critical for ovulation as it produces hyaluronan (HA)-rich extracellular matrix. This HA matrix allows the COCs to lose contact with each other and start to move away from the oocyte through a process called expansion, an essential part of the release of the COCs from the ovary (Chen et al., 1990). The final stages of ovulation involve the disintegration and subsequent rupturing of the ovarian surface closest to the apical region of the Graafian follicle. As the gap-junctions between the oocyte and the adjacent granulosa cells break down, meiotic inhibition is relieved and the oocyte goes on to complete meiosis I. It enters meiosis II and arrests at the diplotene stage of meiosis II as the oocyte is released into the lumen of the oviduct (Richards et al., 1998).

Prior to ovulation, the oocyte produces factors that induce the proliferation of mural granulosa cells and at the same time prevents their differentiation into luteal cells, in a process known as luteinization (Shimasaki et al., 1999, Otsuka et al., 2001, Gilchrist et al., 2004). Once the oocyte has been released, the granulosa and theca cells of the follicle undergo luteinization and form the CL (Richards, 1994, Richards et al., 1998). Luteinization is associated with a rapid loss in cell cycle activators, and a corresponding increase in cell cycle inhibitors (Robker and Richards, 1998b, Robker and Richards, 1998a). This alteration of their inter-cellular balance is believed to stop the proliferation of granulosa cells and induce their terminal differentiation into luteal cells (Richards et al., 1998). The CL synthesises progesterone, which prepares the uterus for pregnancy. In humans, the CL receives signals from the fetus that extend its lifespan, and in turn, it produces the hormones necessary to maintain early pregnancy. If pregnancy does not occur it undergoes luteolysis and regresses (Hoyer, 2010).

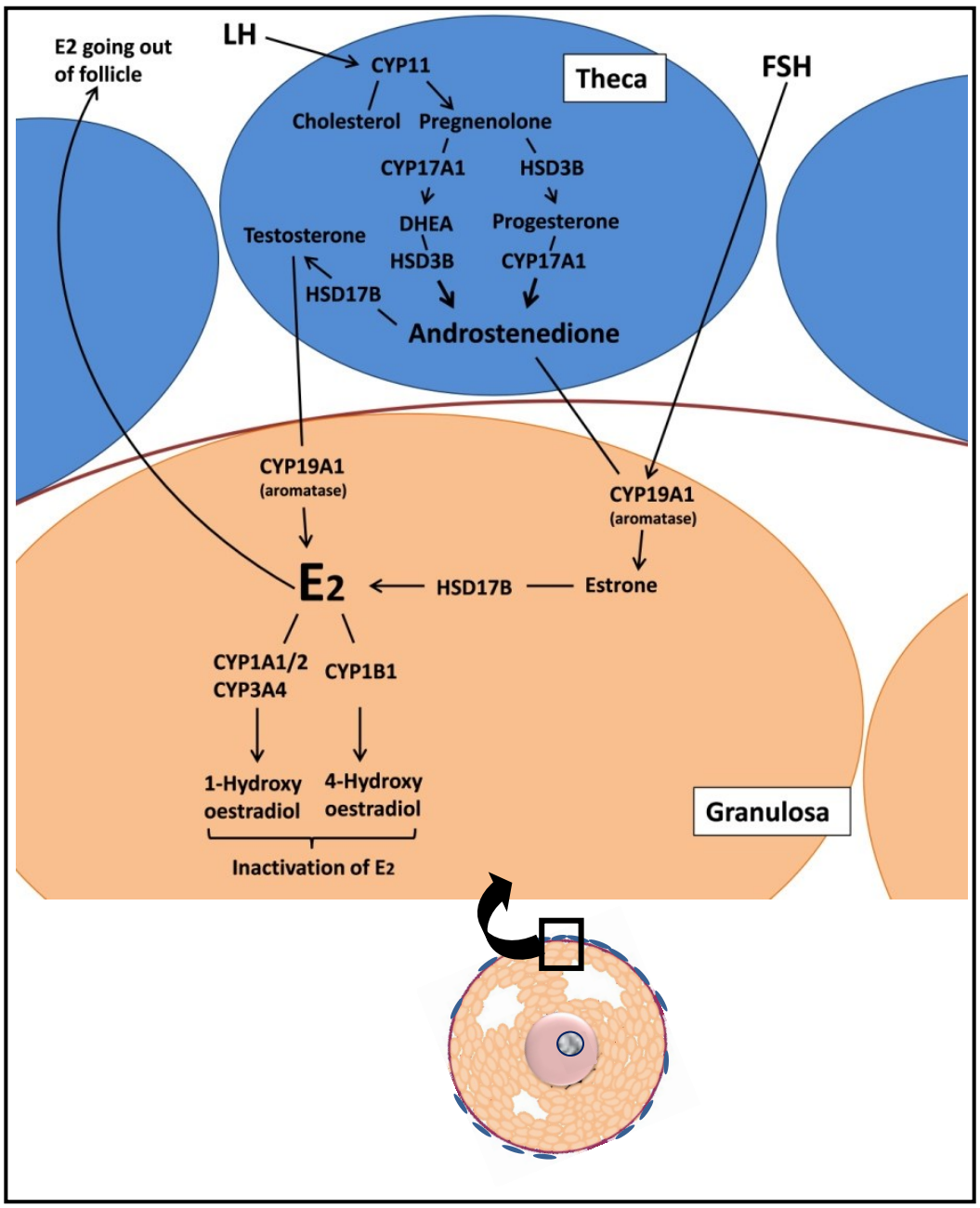


Figure 1.4. Outline of ovarian steroidogenesis.

Ovarian steroidogenesis requires interactions between theca & granulosa cells. LH signalling increases the expression of enzymes required to convert cholesterol to androgens in the theca cells. These androgens get converted into oestrogens in the granulosa cells under the control of FSH signals. The oestrogens can then further be metabolized into inactive metabolites.

1.1.2.5. Follicle atresia

The vast majority of ovarian follicles will never make it to ovulation, but will instead undergo atresia, a process by which follicles degenerate and get re-absorbed. Atresia can occur at any point of follicle development, but the period of germ cell nest breakdown and the pre-antral to antral follicle transition, are two stages associated with particularly high rates of germ cell atresia (Hirshfield, 1991). There are many reasons for follicle atresia. It can occur as a result of low gonatrophin support due to lack of blood supply, due to inhibitory signals from a pre-ovulatory follicle, a deficit in survival factors, or it could be a way of eliminating chromosomally abnormal oocytes.

The main mechanism in follicular atresia is apoptosis, or a form of programmed cell death (PCD) (Rodriguez et al., 2009). During pre-natal development, apoptosis occurs in germ cells, in particular during germ cell nest breakdown (Pepling and Spradling, 2001, Hartshorne et al., 2009), whereas during adult life it can be localised in oocytes as well as in granulosa and theca cells of growing follicles (Hussein, 2005, Rodriguez et al., 2009). Apoptosis is characterised by condensation of the chromatin within the nucleus resulting in its pyknotic appearance, cell shrinkage, fragmentation of the cell, convolutions of the nucleus, production of apoptotic bodies and phagocytosis of these apoptotic bodies.

Apoptosis is driven by various intra-follicular regulators, growth factors, cytokines and steroids. Some factors promote oocyte survival, such as igf1, bcl-2 and interleukin-1β, and other factors are pro-apoptotic, including TGF- β, androgens, bax, p53, TNF, Fas and caspases (Van Nassauw et al., 1999, Tilly et al., 1995, Hu et al., 2001, Hussein, 2005). These factors generate an intricate balance necessary to either initiate or prevent apoptosis.

There are two main mechanisms by which apoptosis is initiated: death receptor-mediated events and mitochondria-mediated events. Death receptor-mediated events occur when a cell initiates the intracellular apoptotic pathway as a direct result to 'death receptors' or to other external factors such as stress, heat, membrane damage

or nutrient deprivation. Death receptors such as Fas, interferon and tumor necrosis factor receptors bind to their ligands and activate apoptotic signals through death domains (DD) or caspase recruitment domains (CARD), which in turn, mediate the activation of pro-caspases and adaptor proteins. This leads to the activation of caspases, triggering apoptosis (Hussein, 2005). Mitochondria-mediated events on the other hand, occur as death signals act through intracellular factors such as pro-apoptotic Bcl-2 proteins, resulting in a release of cytochrome c from the mitochondria. Cytochrome-c and apoptotic protease activating factor 1 (Apaf-1) bind to caspase 9 and in the presence of adenosine triphosphate (ATP), create an 'apoptosome' complex. The apoptosome complex results in the recruitment of cleaved caspase-3, resulting in apoptosis (Hussein, 2005). Other pro-apoptotic factors include Bid, Bad and Noxa (pro-apopotitc BH3-only domain proteins) as well as PUMA (p53 regulated modulator of apoptosis) (Tilly et al., 1995, Hussein, 2005, Kaipa et al., 1997).

In the pre-natal ovary, other types of PCD have also been suggested, namely, autophagy: a lysosomal degradation pathway, and necrosis, consisting of plasma membrane breakdown that results in an inflammatory reaction (Tilly, 2001, Edinger and Thompson, 2004, de Felici et al., 2008, Qu et al., 2007). It is believed that these different mechanisms of PCD create a complex system, that together regulate germ cell and somatic cell death in the developing ovary (Rodriguez et al., 2009).

1.1.2.6 Epigenetic & Genomic imprinting

Epigenetic reprogramming occurs during gamete formation, in a process that induces heritable modifications within the gene activity of a cell, without altering the nucleotide sequences of the DNA strand. Epigenetic changes include DNA methylation, alterations in chromatin structure, nucleosome remodelling and histone modifications (Bonasio et al., 2010). Genomic imprinting works by an epigenetic mechanism that restricts gene expression to only one of the parental chromosomes, but this only occurs in a few hundred of the 25,000 genes in our genome, with the rest being expressed equally. Non-imprinted genes are expressed from both parental genes, and both the maternal and paternal copies of each gene have therefore equal

potential to be active genes. An imprinted gene however expresses only one parental copy and silences the other. It does this by modifying or imprinting one of the chromosomes which then either repels or attracts TFs or mRNA processing factors, altering the expression of the imprinted gene in the process (Barlow and Bartolomei, 2014). A *cis*-acting DNA methylation signal present only on one of the parental alleles, resulting in a molecular mark that leads to the specific expression of the imprinted gene on the marked allele (Jurkowska and Jeltsch, 2013). Genomic imprinting is an effect of inheritance, where the same imprinted gene is active on the same chromosome, either maternal or paternal, regardless of the sex of the offspring (Barlow and Bartolomei, 2014).

During oocyte development, epigenetic reprogramming establishes specific gene imprints which are maintained throughout development in all somatic cells (Jurkowska and Jeltsch, 2013). When two gametes fuse to form a zygote, the epigenetic marks are erased, followed by a period of extensive re-methylation (Hajkova et al., 2002). This is crucial for embryonic development as studies in mice where the male pro-nucleus was replaced with a female, or vice versa, creating a unipaternal cell expressing imprinted genes on both copies of the parental genome resulted in embryos that were not viable. This was because of the presence of imprinted alleles on both chromosomes, resulting in no expression of the allele at all (Surani et al., 1984, Jurkowska and Jeltsch, 2013).

1.2. Toxicology and reproductive function

A considerable number of both man-made and naturally occurring chemicals are considered to be potentially disruptive to the endocrine system for both humans and wildlife (Sharpe and Irvine, 2004, Petro et al., 2012, Rhind et al., 2010). These include environmental toxicants and pharmaceuticals which come from a broad spectrum of chemicals. One group in particular, endocrine disrupting compounds (EDCs), constitute a major focus. EDCs have been described by the United States Environmental Protection agency (USEPA) as agents that ‘interfere with synthesis, secretion, transport, binding or elimination of natural hormones in the body that are responsible for maintenance of homeostasis, reproduction, development and/or

behaviour' (Birklett, 2003). Pharmaceutical and chemical companies produce novel chemicals in the form of new drugs, which can, in some cases, act as EDCs (Rahman et al., 2009). Humans are exposed to thousands of these natural or man-made chemicals throughout their lifespan (Sharpe and Irvine, 2004, Propper, 2005, Woodruff and Walker, 2008, Rahman et al., 2009). Some are ingested as drugs or absorbed through the skin via beauty products such as soaps and perfumes (Rahman et al., 2009), whereas others can leach out of plastic or be inhaled from cigarette smoke or vehicle exhausts). These chemicals can interfere with endocrine mechanisms due to their weak intrinsic hormonal activity, most often by mimicking or inhibiting estrogens through binding to nuclear, membrane, neurotransmitter and/or orphan receptors.

1.2.1 Female reproductive toxicity

Ovarian follicles that undergo growth initiation enter a period of continuous development until they either undergo atresia or develop to the Graafian stage, accompanied by rapid granulosa cell proliferation. That continual growth state, accompanied by the meiotic arrest of the oocyte for up to 45 years in humans, makes them particularly vulnerable targets for environmental toxicants. The somatic cells and the BM of the follicle can be thought of as a protective sheath enclosing the oocyte, but this does not necessarily protect it from the effect of mutagens, directly or indirectly. The majority of toxic compounds are able to access the ovary via the circulation, but if these toxicants are able to pass through the BM, then the oocyte can also potentially be affected. Any compound that has a direct effect on the ovary, may also be able to alter epigenetic mechanisms in the oocyte, resulting in trans-generational epigenetic effects (Newbold et al., 2000, Miller et al., 2004, Jefferson et al., 2007, Bernal and Jirtle, 2010, Zama and Uzumcu, 2010). Even if such chemicals only have a direct effect on granulosa or thecal cells, they are still able to affect the oocyte indirectly.

1.2.2. Some examples of mechanisms of action of toxic agents on the ovary

Pre- and postnatal ovaries contain large numbers of germ cells and follicles at various stages of development. Pre-natally, this includes the rapid proliferating PGCs and the oocytes undergoing the first meiotic division. The mature ovary contains primordial follicles with oocytes in meiotic arrest, dividing granulosa cells and maturing oocytes of growing follicles, as well as ovulating oocytes resuming meiosis. The ever-changing environment is a major challenge for reproductive toxicity studies, as follicles in different stages of growth may well vary in their susceptibility to different compounds. A compound might, for example, only target growing follicles but have no effect on the primordial follicle pool, or vice versa.

Compounds targeting the primordial pool can have adverse effects on fertility, arguably more so than those targeting growing follicles. If the primordial pool of follicles is damaged, future follicle growth and ovulation may be affected. At worst, a chemical that interferes markedly with the resting pool could result in premature ovarian insufficiency (POI) (De Vos et al., 2010). In contrast, if a compound specifically targets growing or pre-antral follicles, perhaps by targeting dividing granulosa cells, these follicles might undergo atresia which could result in cyclic disturbances for the few months following exposure to the compound: however, once the compound and its effects are removed, new follicles (from the unaffected resting pool) will begin growing and form normal ovulatory follicles, thus restoring fertility (Cortvrindt and Smitz, 2002). Sustained effects on growing follicles can, though, have long-term consequences: studies into the effects of chemotherapy on the ovary are providing growing evidence that repeated damage to growing follicles can have a severe effect on the primordial pool, as the loss of growing follicles leads to premature activation of primordial follicles and consequently, a depletion of the primordial follicle pool (Meirow et al., 2010, Morgan et al., 2012, Kalich-Philosoph et al., 2013, Morgan et al., 2013).

There are several other ways in which chemicals could disrupt oocyte development. The pre-natal phase of ovary development is considered a particularly vulnerable phase, as various chemicals have been shown to affect fertility when exposure occurs

during fetal development (Fowler et al., 2008, Bellingham et al., 2012, Matikainen et al., 2002, Hunt et al., 2012). During meiotic progression of the oocyte, chromosomes utilise a bipolar spindle for their segregation for both meiotic divisions. If disturbed, this could lead to impairment in chromosome pairing or spindle formation, resulting in non-disjunction (Fragouli et al., 2011). Regulation of progression of the cell cycle is tightly controlled by feedback mechanisms that sense disturbances and by checkpoint controls that protect the cell from such errors and ensure that aneuploidy is prevented (Vogt et al., 2008). Failure during these meiotic checkpoints can result in meiotic errors, and the resulting mutations introduced to the genetic material have the potential to be passed on to the subsequent generation. Studies have been carried out on the possible effect of environmental chemicals on meiotic disturbances (Can and Semiz, 2000, Hunt et al., 2003, Hunt et al., 2012) and have illustrated that the chemicals interfere with the actions of estrogen receptors (ERs) (Susiarjo et al., 2007) and cause abnormalities in the alignment of chromosomes and spindle formation (Can and Semiz, 2000, Eichenlaub-Ritter et al., 2008, Hunt et al., 2012). E2 inhibits germ cell nest breakdown and protects oocytes from programmed cell death with binuclear oocytes and multioocyte follicles (MOFs) reported as a result of estrogenic compound exposure (Pepling and Spradling, 2001, Kim et al., 2009). MOFs are often used as an indicator of an adverse effect, as they are considered a likely result of disruption to germ cell nest breakdown (Nagao et al., 2001, Suzuki et al., 2002, Jefferson et al., 2007, Kim et al., 2009, Cimafranca et al., 2010, Rivera et al., 2011, Karavan and Pepling, 2012). Although the vast majority of oocytes affected by chemicals are likely to end up becoming atretic (Rodriguez et al., 2009), some might form aneuploid ovulated oocytes, with the consequent potential of an aneuploid embryo and likely miscarriage.

Reproductive toxicants might either target the oocyte specifically, or have more general effects on the surrounding somatic cells. In either scenario, reproductive disorders can occur. The follicle is a complex structure relying on interactions between the oocyte and its somatic cells (Eppig, 1979, Gilchrist et al., 2004, Su et al., 2004, Thomas and Vanderhyden, 2006, Orisaka et al., 2009). Granulosa and theca cells are responsible for hormone production within the ovary as well as controlling

the release of oocytes throughout the adult reproductive lifespan. This is mainly regulated through the expression of autocrine and paracrine factors, creating intricate feedback loops within the follicle that are essential for normal follicle development and for meiotic competence of the oocyte. This complex communication network formed by the oocyte, granulosa cells and theca cells, together drives follicle development. Any chemical affecting either the oocyte or the surrounding somatic cells can lead to a disruption in the secretion of growth factors, then affecting the oocyte-granulosa cell regulatory loop (Su et al., 2004). These chemicals could also interfere with the feedback loop between the ovaries and pituitary gland to perturb the balance of the hypothalamo-pituitary-gonadal axis. Disruption could not only have negative effects on follicle development, oocyte maturation and ovulation, but could also significantly affect the production of ovarian hormones from the ovary (Canipari, 2000).

1.3. In vitro ovary and follicle culture systems

The first successful rodent ovary culture was established in 1937 (Martinovitch, 1938, Martinovitch, 1937), and subsequently, various culture methods have been developed with the aim of growing ovaries and follicles from an immature state to fully mature, fertilizable oocytes (Blandau et al., 1965, Eppig and Schroeder, 1989, Spears et al., 1994, Eppig and O'Brien, 1996, Klinger and Felici, 2002, Obata et al., 2002, O'Brien et al., 2003, Picton et al., 2003, Xu et al., 2009, Jin et al., 2010). Ovary and follicle cultures have now become a widely used tool to study the development of follicles in reproductive biology and toxicology and have been successfully established in humans, primates, cattle, sheep, pig, mouse, and rat (Roy and Treacy, 1993, Hirao et al., 1994, Ralph et al., 1995, Telfer et al., 2008, McLaughlin and Telfer, 2010, Xuying et al., 2011).

In 1989, the first *in vitro* pre-antral follicle culture that led to the birth of live mouse pups was established (Eppig and Schroeder, 1989). The real challenge, however, has been to establish a culture system that allows for the expansion and development of the germline progenitor cell right through both meiotic divisions to produce a mature fertilizable germ cell that is then capable of producing live offspring. A few studies

have managed to create the culture conditions necessary to produce live pups from immature cultured follicles (Eppig and Schroeder, 1989, Spears et al., 1994, Eppig and O'Brien, 1996, Obata et al., 2002, O'Brien et al., 2003, Jin et al., 2010, Mochida et al., 2013) but many were invasive, requiring major manipulation to the follicles. For example, Obata *et al* (2002) used fetal ovaries as starting material, culturing pre-meiotic female germ cells from E12.5 embryos to antral oocytes, although the culture system required transfer of nuclei of the E12.5 germ cells into enucleated grown oocytes from adult mice following 28 days in culture. This allowed the oocytes to resume and complete meiosis *in vitro,* and following *in vitro* fertilization they successfully developed into blastocysts, with living pups obtained following embryo transfer (Obata et al., 2002).

1.3.1. In vitro ovary culture in reproductive toxicity testing

Exposure of reproductive toxicants can occur in the form of individual chemicals or as chemical mixtures and it has proven difficult to establish which situation causes more harm to reproductive function, in particular because the effects might not become evident until years later (Sharpe and Irvine, 2004). Furthermore, different periods of vulnerability to exposed compounds mean that a fetus might not be affected by a chemical in the same way as an adult would, making the testing of such chemicals on reproductive function difficult, yet that much more crucial.

Many *in vivo* reprotoxicity studies, in particular those carried out on the reproductive effects of pharmaceuticals, use end-points such as pregnancy, implantation and number of offspring, parameters that do not identify any potential effects on the primordial follicle pool. Consequently, *in vivo* study designs used at present might not pick up long-term effects on the primordial follicle pool since it may not affect immediate ovulation rates and subsequent pregnancies, but might have longer term consequences on reproductive lifespan. On the other hand it is also possible that an effect seen in the neonatal ovary may, in fact, correct itself in later life (Bristol-Gould et al., 2006).

In vitro ovary and follicle culture models allow for the possibility of varying culture parameters in a highly controlled manner, and thus have the potential to allow a more thorough evaluation of early-ovary development and for reproductive toxicity studies, than do *in vivo* studies alone. *In vitro* cultures used in reproductive toxicity studies allow for a thorough investigation of the mechanism of action of toxicants and how they contribute to oocyte or somatic cell damage. They also allow for a detailed analysis of oocyte quality, effects on the establishment of the primordial follicle pool, and paracrine interactions (Sun et al., 2004). Culture systems have the potential to reveal whether the ovary is directly targeted by the toxicants, with effects observed *in vivo* but not *in vitro* presumably being indirect. The cultures can also reveal if compounds target follicles at specific stages of development and can give deeper insight into the way toxicants might affect the chromosomal integrity of the oocyte, or if they have the ability to alter hormonal signalling within and/or between follicles.

Testing of chemicals *in vivo* is time consuming and costly. To date, *in vitro* models have been used primarily as a preliminary or secondary screening protocol for toxicity testing. The lack of an alternative test system to available *in vivo* study designs has been commented on (Davila et al., 1998, Jackson, 1998, Cortvrindt and Smitz, 2002), leading to an increased demand for adequate *in vitro* models that may be used to gain an insight into the mechanisms of chemical exposure and pinpoint potentially hazardous products on reproductive function. *In vitro* studies might provide a good way forward to investigate the direct effects of certain chemicals on primordial germ cell reservoir in the ovary.

1.4. Topoisomerase-II

Type II topoisomerases (Topo II) are large homodimeric nuclear enzymes that play a role in the chromosome condensation and in the separation of sister chromatin during mitosis. Due to the double stranded nature of DNA, topological problems can arise during transcription and replication when a DNA strand needs to be unwound, as this can result in overwinding, or supercoiling, of other areas of the DNA molecule. Topo II regulates the topology of DNA during mitotic divisions by creating transient

double (type II) DSBs in the DNA, and thereby relieving the torsional stress created by supercoiled DNA strands with superhelical turns (Fig. 1.5) (Roca, 2009, Nitiss, 2009). They are also thought to be required for prophase I of meiosis, during the untangling of sister chromatids following recombination, as well as for the segregation of replicated chromosomes (Nitiss, 2009, Russell et al., 2000, Li et al., 2013). Furthermore, supercoiled areas are associated with an increase in recombinational events due to genetic instability (Baguley and Ferguson, 1998) and this may generate duplications, deletions, insertions, inversions or substitutions within a DNA sequence. Therefore, topoisomerases are also believed to play a role in reducing these recombination rates by relieving the torsional stress (Baguley and Ferguson, 1998).

Topo II acts by breaking one DNA strand with each subunit, passing the unbroken strand through the break and then resealing it. Mammals express two types of Topo II paralogues: Topo IIα and Topo IIβ, and although they share 70% amino acid similarity and have near identical catalytic activity (Li et al., 2013), they have different functions and expression patterns (Kimura et al., 1994). Topo IIα expression peaks at the G2/M stage of the cell cycle whereas Topo IIβ expression is not regulated by the cell cycle (Woessner et al., 1991, Capranico et al., 1992). In the mouse ovary, Topo IIβ is expressed in oocytes at all developmental stages. Its expression is low in granulosa cells of primordial follicles but becomes more pronounced in the granulosa cells of primary and secondary follicles, whilst it becomes abundant in the granulosa cells of pre-ovulatory follicles (Zhang et al., 2013). Within the germ cells nucleus, Topo II is localised within the germinal vesicle (GV) prior to meiotic entry, but once GV breakdown (GVBD) has occurred, it becomes localized on the chromosomes, adjacent to the centromeres and along the chromosome arms (Li et al., 2013). The expression pattern of Topo IIα within the ovary has yet to be reported.

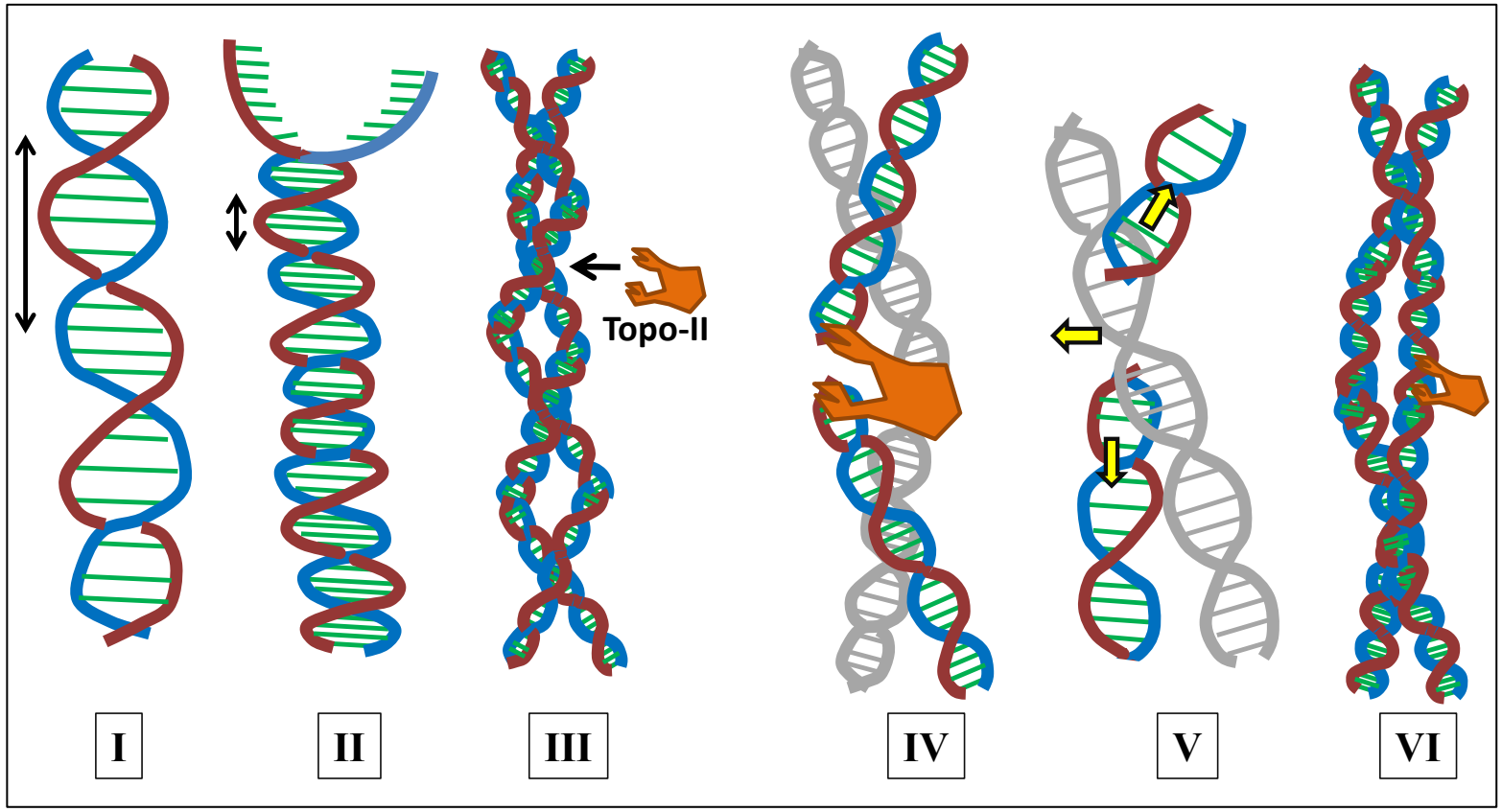


Figure 1.5. Action of Topo II. A DNA strand is in its 'relaxed' state (I). During replication, unwinding of parts of the DNA strand increases the twist strain on other areas of the strand, resulting in over-winding (II). If further topological strain is applied, the double helix crosses over on itself, resulting in a supercoiled strand (III). Topo II creates breaks in both DNA strands (IV) so that the unbroken strand can pass through, relieving the topological stress (V). Topo II then religates the broken strand (VI).

1.4.1 Topoisomerase-II knockout models

Akimitsu *et al* (2003) demonstrated the importance for Topo IIα in development with the generation of a Topo IIα knockout (-/-) mouse, whose embryos were unable to progress beyond the 4-8 cell stage (Akamitsu et al., 2003). Conditional knock-out models have therefore been adapted to study any loss-of-function effects associated with Topo IIα but none investigated the effect of Topo IIα on fertility (Johnson et al., 2009, Carpenter and Porter, 2004). Topo IIβ knockout (-/-) mice however, are not embryonic lethal but instead develop defects in motor axons and in the stratification of the cerebral cortex (Lyu and Wang, 2003, Yang et al., 2000). Zhang *et al* (2013) generated a conditional Topo IIβ knockout model where Topo IIβ was deleted in granulosa cells. This resulted in a rise in granulosa cell DNA damage, leading to increased follicle atresia in these ovaries. Therefore they concluded that the Topo IIβ enzyme plays an essential role in granulosa cells where it prevents the accumulation of DNA damage and apoptosis (Zhang et al., 2013).

1.4.2 Topoisomerase-II inhibitors

Due to its role in cell division, inhibiting Topo II has been a popular method for targeting tumour cells during cancer treatments. Doxorubicin, ICRF-193, teniposide and etoposide (VP-16) are all Topo II poisons that act by inhibiting Topo II from religating DNA molecules by interfering with DNA processing. Most Topo II inhibitors inhibit the enzymatic activities of both Topo IIα and Topo IIβ (Li et al., 2013). Topo II inhibition generates high numbers of DSBs, activates DNA damage checkpoints and can result in chromosomal abnormalities leading to fragmentation of DNA and results in tumour cell death (Roca et al., 1994, Damelin and Bestor, 2007, Montecucco and Biamonti, 2007). However, due to the highly toxic nature of these drugs, they can also result in adverse side-effects on fertility (Anderson and Berger, 1994, Soleimani et al., 2011, Ben-Aharon et al., 2010). Inhibition of Topo II during mitosis and meiosis can result in an incomplete separation of chromatids and chromosomes, and consequently, genomic mutations can occur (Baguley and Ferguson, 1998). In male mice, etoposide interferes with the early stages of prophase I, specifically between leptotene and diakinesis (Russell et al., 2000). Etoposide inhibits the religation of the DNA strand following the cut made by Topo II, and

exposure of mouse spermatocytes during early to mid pachytene results in locus deletions and a reduction in crossovers. Interestingly, spermatocytes undergoing meiosis II are much less sensitive to Topo II inhibition than they are whilst during meiosis I (Russell et al., 2000, Kallio and Lahdetie, 1996). Similar effects have been reported in the female mouse, where oocytes from dictyate to diakinesis were affected by Topo II inhibitors, although unlike in the male, they were also highly sensitive to Topo II inhibition during the second meiotic division (Tateno and Kamiguchi, 2001a). One of the reasons for this difference in susceptibility between male and female germ cells could be that Topo II inhibitors have increased accessibility to the DNA in oocyte due to the diffuse state of chromosomes at meiotic arrest, compared with the more condensed chromosomes in male germ cells (Baguley and Ferguson, 1998).

1.4.2.1 AZTC

The majority of the work carried during the PhD concentrated on one type of compound, an antibacterial agent that was under pharmaceutical development at AstraZeneca: 'Astrazeneca Test Compound' (AZTC). AZTC is a Topo II inhibitor that targets bacterial Topo II, which has mammalian homologues possibly involved with meiosis. Previously it was noted in male rats exposed to this compound that there were effects on spermatogenesis, namely, testicular degeneration, abnormal sperm morphology and lowered sperm counts (unpublished data, AstraZeneca). The testicular damage caused is likely reversible in the testis and would potentially be manageable and recoverable in the clinic, however if a female fetus were to be exposed *in utero,* this could potentially irrevocably damage the pool of surviving oocytes. AZTC was therefore selected to investigate the efficacy and validity of ovarian culture methods.

1.5. Aims of PhD

In the past 50 years, various culture methods have been developed with the aim of growing ovarian follicles in vitro. These culture systems have become a widely used tool in reproductive biology and toxicology, but the real challenge has been to

establish a culture system whereby mouse germ cells can be cultured from a pre-meiotic stage to a mature oocyte. The pre-natal period of oocyte development is an important one, as it results in the formation of the primordial follicle pool. A woman's reproductive span is dependent on the size of the primordial follicle pool, which is determined during fetal development, when the germ cell pool undergoes proliferation and enters prophase I of meiosis. Furthermore, in the pharmaceutical industry, the majority of reproductive toxicity testing is carried out *in vivo* to assess the effects of a compound for inclusion of women of childbearing potential in clinical trials. This involves using immediate end-points such as ovulation, pregnancy, implantation and number of offspring, parameters that do not identify any potential effects on the primordial follicle pool. Consequently, *in vivo* study designs used at present might not pick up long-term effects of chemicals the primordial follicle pool since it may not affect immediate ovulation rates and subsequent pregnancies, but could have longer term consequences on reproductive lifespan. This has led to an increased demand for adequate *in vitro* models that may be used alongside *in vivo* methods, to study the biology of the pre-natal ovary and to gain an insight into the mechanisms of pre-natal ovarian toxicants.

The aims of this PhD were to:

1. Bridge, adapt and improve existing culture methods to generate a culture system that would span meiotic entry to meiotic arrest, germ cell nest break-down, follicle formation and initiation of follicle growth.
2. Investigate the effects of a novel Topo II inhibitor developed by AstraZeneca, AZTC, on the pre-natal and post-natal ovary. The aim was then to compare the *in vitro* effects of exposure to the Topo II inhibitor pre-natally, in part, using the embryonic ovary culture system developed above, to effects from *in vivo* studies.

Chapter 2.
Materials and Methods.

2.1 Neonatal ovary culture

2.1.1. Dissection medium

The dissection medium consisted of Liebovitz (L-15) medium (Invitrogen, 11415049). Before use the medium was first adjusted to the osmolarity of 285 mmosmol using the calculation below, and adding water (Sigma, W1503):

Difference/actual x volume = volume of H_2O required

The medium was supplemented with 3 mg/ml BSA (Sigma, A3311) and once dissolved, it was filter sterilised using Becton-Dickson syringes and Iwaki filters (Iwaki, 2032-13 or 2052-025). 1-2 ml of the prepared dissection media was placed in embryo dishes (VWR, 720-0579) and warmed to 37˚C before dissected ovaries were placed into the dishes.

2.1.2. Culture medium (simple medium)

The culture medium was α-MEM (Invitrogen, 22571020). Before use it was adjusted to the osmolarity of 285 mmosmol by adding water using the above calculation (section 2.1.1). The medium was supplemented with 3 mg/ml fatty acid free BSA (Sigma, A8806) and filter sterilised as above (section 2.1.1). 1 ml of media was required per ovary, and media was incubated for a minimum of 30 minutes in plastic culture tubes with dual-position caps or in the culture plate, allowing for equilibration of media prior to ovary culture.

2.1.3. Plate Preparation

Flat bottom 24 well culture plates were used for this culture (Greiner, 662960). 1 ml of culture medium was placed into each well and a nucleopore polycarbonate membrane (Whatman, 110414) was placed on top using sterilised tweezers. The plate (without the lid) was placed in a laminar flow hood under UV light for 30 minutes for sterilisation prior to culture.

2.1.4 Tissue collection & ovary culture

Neonatal mouse pups were culled by decapitation and pinned to a corkboard in a flow hood. A ventral incision was made and ovaries, with the ovarian bursa attached, were dissected under a microscope. The ovaries were placed in petri dish containing the pre-prepared dissection medium (section 2.1.1). Using insulin needles, the

ovaries were dissected free of the ovarian bursa and extra tissues under the microscope in the flow hood. The ovaries were transferred onto membranes in the culture wells using a coated drawn Pasteur pipette. Ovaries were cultured for 6 days. The culture medium was changed every other day by exchanging 500 µl of the medium for 500 µl of freshly prepared, pre-equilibrated medium using a pipette. This culture medium will be referred to as the simple medium for this point onwards.

2.2. Histology

2.2.1 Ovary fixation and agar embedding

Following culture, ovaries were washed in 1xPBS for 5 minutes and then fixed either in Bouins fixative for 1-2 hours, or in 10% buffered formalin overnight (Sigma, HT5014). Ovaries were washed once in 70% ethanol to remove any remaining fixative, and then placed in fresh 70% ethanol for storage before processing. 2% agar (Sigma, A1296) was made up in ddH20 and ovaries were placed in a drop of agar for support and ease of handling.

2.2.2 Wax embedding

Ovaries (in agar blocks) were placed in tissue processing cassettes and then passed through a series of alcohol solutions of increasing concentration (70%, 90%, 100% x2), before being immersed in a paraffin wax and xylene solution, allowing the ovarian tissue to become penetrated by the molten paraffin wax. They were then placed in truncated plastic moulds (Park Scientific), which were subsequently filled with wax and allowed to cool.

2.2.3 Sectioning and mounting

Wax blocks were removed from the moulds and the base trimmed down to create a flat surface. A heated chuck was fused to the base of the block and together, and placed in water to cool the wax and secure the block onto the chuck. The edges of the block were then trimmed around the tissue with a razor blade before being fixed onto a microtome. The block was positioned and orientated so that the surface of the block is cut at a right angle and the tissue was sectioned at 5 µm. Wax ribbons were floated onto a warm water-bath (approx. 42˚C) and carefully placed onto poly-L-lysine

coated slides (Fisher), which were placed in a slide-rack and left to dry overnight in a 37˚C oven.

2.2.4 Haematoxylin and eosin staining

Slides were placed in a staining rack and placed in xylene for (10 minutes x2) for dewaxing before being re-hydrated up through a series of decreasing ethanol concentrations (100%, 95%, 90%, 70%), being left for 5 minutes at each concentration. If the ovaries had been fixed in bouins, the slides were then placed in 70% ethanol lithium carbonate for 5 minutes to remove any trace of bouins from the tissue. Slides were then washed in tap water for a further 5 minutes and placed in freshly filtered haematoxylin for 2-3 minutes to stain the nuclei, before then being placed back in the tap water. The intensity of the stain was checked under a microscope and if the stain was too dark then the slides were briefly dipped into acid alcohol and the stain was checked again. Once the desired intensity of stain was achieved, the slides were placed in Scott's tap water solution (STWS) for 3 minutes, followed by a 3 minute was in tap water. The slides were then placed in filtered eosin stain for 2 minutes, washed in tap water, and then placed in potassium alum for a further 2 minutes to fix the stain, followed by a rinse in tap water. The staining was then checked under a microscope. If the desired intensity of stain was observed then the slides were dehydrated through the ethanol solutions (70%, 90%, 95%, 100%x2), but left slightly longer in the ethanol solutions if the stain was too dark. The ovary sections were then cleared in xylene for 5 minutes, covered with a xylene-based mountant (DPX) and coverslips placed on top, avoiding air bubbles. The slides were left overnight in a fume hood and once dry, they were examined and imaged under a bright-field microscope (DMLB microscope, DFC480 camera and IM50 software, Leica).

2.3 Follicle counts and classification

2.3.1. Follicle counting

Following haematoxylin and eosin staining, every 'n'th ovary section of the ovary was photographed under bright-field microscopy (described in Section 2.2.4). Each figure was converted to a JPEG file using Corel Photo Paint, reducing the size of the

file without compromising visual quality. The figure was opened in ImageJ for follicle analysis, which was carried out using the cell counter tool in imageJ. Every 6th ovary section was counted from neonatal (P0 and P4) and embryonic ovary cultures (described in Chapter 4), whereas from the *in vivo* rat study, every 20th was counted from the PND 5 ovaries, every 40th from PND 15 ovaries, and finally, every 50th from the adult rat ovaries (described in Chapter 3). The frequency of sections analysed were selected in relation on ovary size, with every 6th section selected for the smallest mouse ovaries whereas every 20th-50th were selected for the larger rat ovaries, as taking every 6th would requiring an analysis of a very large number of sections, whereas counting every 20th, 40th or 50th should give a representative sample of follicles in the larger rat ovaries.

2.3.2. Follicle classification

Follicles counted were categorised into the following follicle types: Primordial follicle (Type 1: PMF), an oocyte with a flattened layer of granulosa cells, transitional (Type 2: TRNS), an oocyte with some flattened and some cuboidal granulosa cells, primary follicle (Type 3: PRIM), when all the granulosa cells in the layer have become cuboidal, Secondary (Type 4: SEC), once a second layer of granulosa cells has formed, pre-antral (Type 5; PreA), when the oocyte is surrounded by more than two layers of granulosa cells but without an antrum, early Antral (Type 6: EarlyA), follicles with a small antrum, and finally, Late Antral (Type 7: LateA) follicles with a large antral cavity (See Figure 2.1). Follicle health was recorded onto a separate sheet, where oocytes containing a shrunken and pyknotic nucleus or granulosa cells, identified by a dark eosin stain, were classified as unhealthy, see below. Only follicles with a visible nucleus were included in the counts to avoid double-counting of follicles. The analysis was always carried out blind to the treatment type.

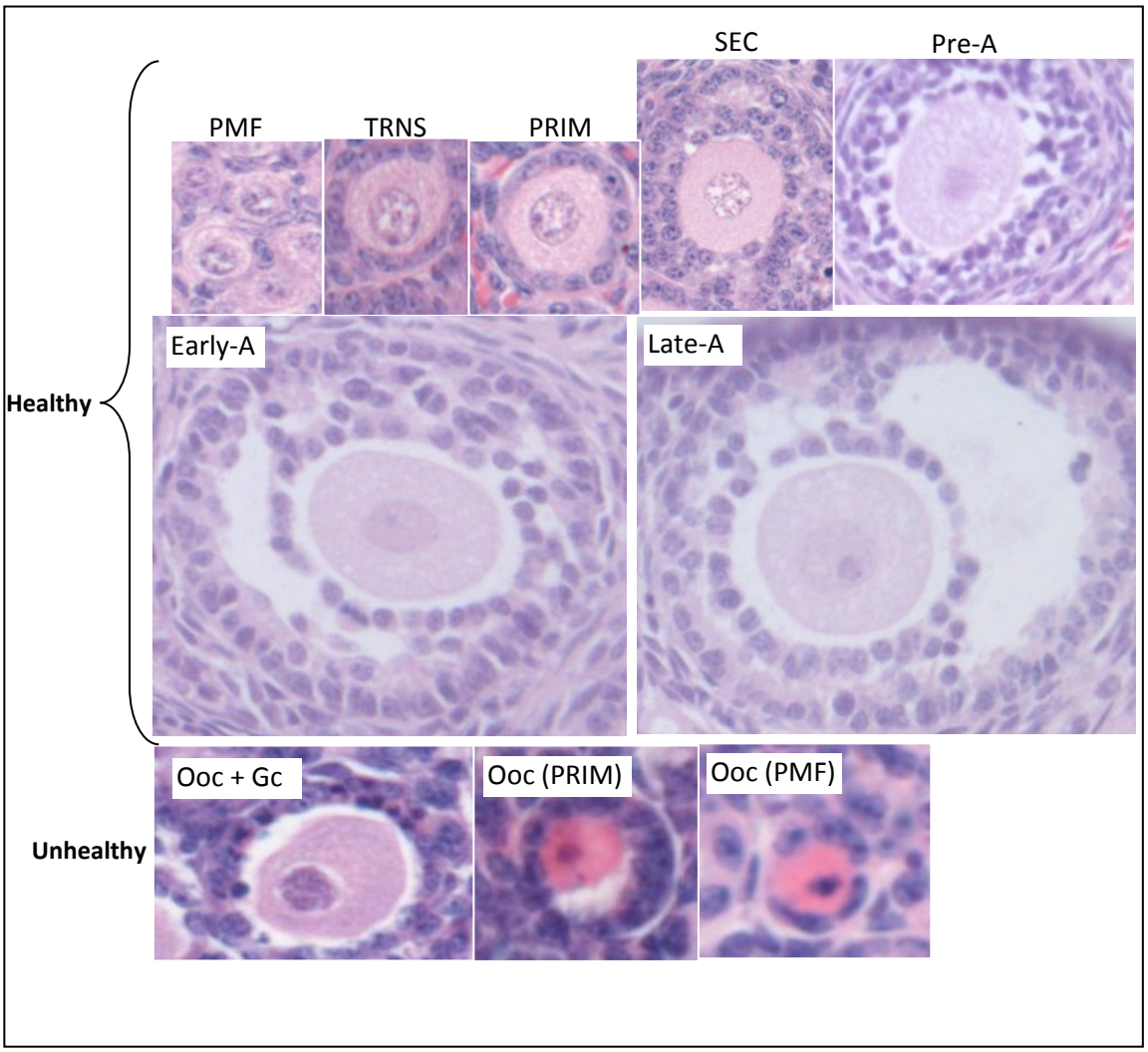


Figure 2.1. Classification of ovarian follicles according to follicle stage and health. Ovarian follicles were counted and categorised into their repsective stages of follicle growth: primordial (PMF), transitional (TRNS), primary (PRIM), secondary (SEC), early antral (Early-A) and late antral (Late-A). Follicle health was also measured and follicles with an unhealthy oocyte, identified by a darker eosin stain and a pyknotic nucleus, or with unhealthy pyknotic granulosa cells, or both.

2.3.3. Correction of follicle counts

The size of an ovarian follicle is larger than the thickness of the sections and this increases the chance of the same follicle being double counted in two nearby section. This means that when calculating the final estimate over total follicle numbers per ovary, the final number will be an over-estimate. Here, in order to get more reliable estimates of the total number of follicles within an ovary, the Abercrombie equation was used (Abercrombie, 1946). The Abercrombie equation calculates a more accurate density of a population of cells by applying a correction factor to the total number of counted nuclei. It requires a calculation of the mean diameter of nuclei from each follicle type, which are then incorporated into the calculation along with the thickness of the section, to estimate the total number of cells. The Abercrombie equation is outlined below:

$$\text{Average number of nuclear points per section} = \mathbf{A} \times \left[\frac{M}{L + M} \right]$$

A: crude count of the number of nuclei counted in section

M: thickness of section

L: Average length of nuclei

The mean diameter of nuclei (L) for each of the follicle types was measured, with a minimum of ten examples for each follicle stage used to obtain the mean. This number was then used to calculate the value within the brackets above, where the thickness of the section (M) was 5 μm. The results are outlined in Table 1. This was then multiplied with the crude count of nuclei in the section and with the selected ' number of sections that were used for counting to calculate the end approximate number of nuclei in the whole ovary.

Table 2.1.Correction factor. The obtained values for each follicle type, calculated from the thickness of section (M) and average length of nuclei (L) using the

Follicle Type	$\frac{M}{L+M}$
Primordial	0.34544
Transitional	0.30444
Primary	0.27661
Secondary	0.26657
Pre-Antral	0.25954
Early-Antral	0.24478
Late-Antral	0.24469

2.4 Immunohistochemistry

2.4.1 Dewaxing and rehydration

Slides were dewaxed in xylene (2x5 minutes) followed by rehydration through a series of decreasing ethanol concentrations (100% 2x5 minutes, followed by 95%, 90% and 70% for 5 minutes each) before rinsing in ddH_2O for 5 minutes.

2.4.2. Antigen retrieval

Slides were placed in a plastic slide holder filled with citrate buffer (0.01M) and covered with cling film. They were microwaved for 4x5 minutes, and the slide holder was topped up with 50 ml of buffer in between to ensure that the slides were well covered. The antigen of interest becomes masked by the extensive cross-linking of proteins during tissue fixation and processing, but antigen retrieval uses heat to un-fold the proteins within the tissue, exposing the epitope and therefore permitting successful binding of the antibody to the antigen. The slides were then left to stand in the citrate buffer for a minimum of 20 minutes, until cooled.

2.4.3 Blocking

In order to prevent any non-specific antibody binding and background staining, endogenous peroxidases in the tissue were blocked with 3% H_2O_2 in Methanol for 30 minutes. The slides were then washed in PBS/0.1% Triton X (Sigma, TT9284) for 5 minutes, twice and placed in a wet chamber: a shallow, plastic box with wet tissue

paper in the bottom to prevent dehydration. The area around the tissue was dried and a PAPpen was used to draw around the tissue sections. To further reduce any non-specific reactions, slides were then blocked with a solution of 20% Normal Goat Serum supplemented with 5%BSA and PBS (NGS/BSA/PBS solution: 2ml Serum, 0.5g BSA, 8ml 1xPBS) and left in the wet chamber for one hour at room temperature.

2.4.4 Primary antibody

The primary antibody was diluted in the NGS/BSA/PBS solution (sections 2.4.3 & 2.4.4). The blocking solution was tipped off the slides, 100 µl of the primary antibody solution was then applied to each slide and they were left in the wet chamber overnight at 4˚C.

2.4.5 Secondary antibody

Primary antibody solution was tipped off the slides and they were washed in dH_2O for 5 minutes, followed by two more 5 minutes washes in PBS/0.1% Triton X. The secondary antibody (biotin conjugated) was diluted in the NGS/BSA/PBS solution (Section 2.4.5) and was applied at 100 µl per slide and left for one hour at room temperature in the wet chamber.

2.4.6 Antigen detection

Serum was tipped off the slides and they were washed in dH_2O for 5 minutes, followed by two more 5 minutes washes in PBS/0.1% Triton X. The secondary antibody was detected with the help of the Vectastain ABC Elite kit (Vector laboratories, PK6100). The avidin-biotin complex (ABC) kit utilises the high affinity of avidin for biotin. The biotinylated secondary antibody binds to the primary antibody, which in turn is bound to the protein of interest. The biotin-binding sites bind to the biotinylated secondary antibody, resulting in more enzyme being attached to the target than if only using an enzyme-conjugated secondary or primary antibody. Two drops of solution A and two drops of solution B from the ABC kit were placed into 5ml of PBS and left for 30 minutes before use. Around 4 drops of the ABC solution were applied to each slide and left for 30 minutes in the dark at room

temperature. The slides were then washed in PBS/0.1% Triton X for 5 minutes, twice.

The ABC complex was visualised with the help of 3,3'- diaminobenzidine (DAB) (Vector Laboratories, SK4100), which is the substrate for the peroxidase enzyme, resulting in dark brown staining. From the DAB kit, one drop of buffer, two drops of DAB and 1 drop of hydrogen peroxide (added immediately before use) were added to 2.5 ml of water to make up the DAB. The solution was wrapped in foil and added to the slides. The slides were left for about 1 minute, or until brown staining became visible under the microscope. To stop the reaction, the slides were washed in ddH_2O. They were then washed several more times in ddH_2O before being placed in haematoxylin for 30 seconds to counter-stain the nuclei. The slides were then rehydrated up through a series of ethanol solutions (70%, 90%, 95%, 100%x2, around 30 seconds in each), placed in mounting xylene for 5 minutes and coverslipped using DPX mountant. Once dry, the sections were visualised as previously described (see Section 2.2.4).

Chapter 3.
The effect of AZTC on the pre-natal rodent ovary *in vivo*

3.1 Introduction

Humans are exposed to thousands of man-made chemicals throughout their lifespan (Sharpe and Irvine, 2004, Propper, 2005, Woodruff and Walker, 2008, Rahman et al., 2009). Pharmaceutical and chemical companies continually produce novel compounds in the form of new drugs or environmental chemicals, which can in some cases interfere with the endocrine and/or reproductive systems (Rahman et al., 2009). Therefore rigorous reproductive toxicity testing of such pharmaceuticals has been set in place within the pharmaceutical industry.

The thousands of new compounds produced by the pharmaceutical industries require extensive testing for pharmacological activity and toxicological effects. Potential toxic effects are assessed throughout the developmental process of a compound where histopathological assessments of ovaries is conducted prior to reproductive toxicity testing in the repeat toxicity studies (Fig. 3.1) In the pharmaceutical industry, currently, *in vivo* reproductive toxicology tests are carried out relatively late in the drug development process, during the phase II clinical trials and development phase of a drug. Reproductive toxicity must cover both fertility and pre-natal developmental effects. Furthermore, analysis into effects on sexual behaviour, oestrous cycling, spermatogenesis, fertilization and implantation must be taken into consideration (Cortvrindt and Smitz, 2002).

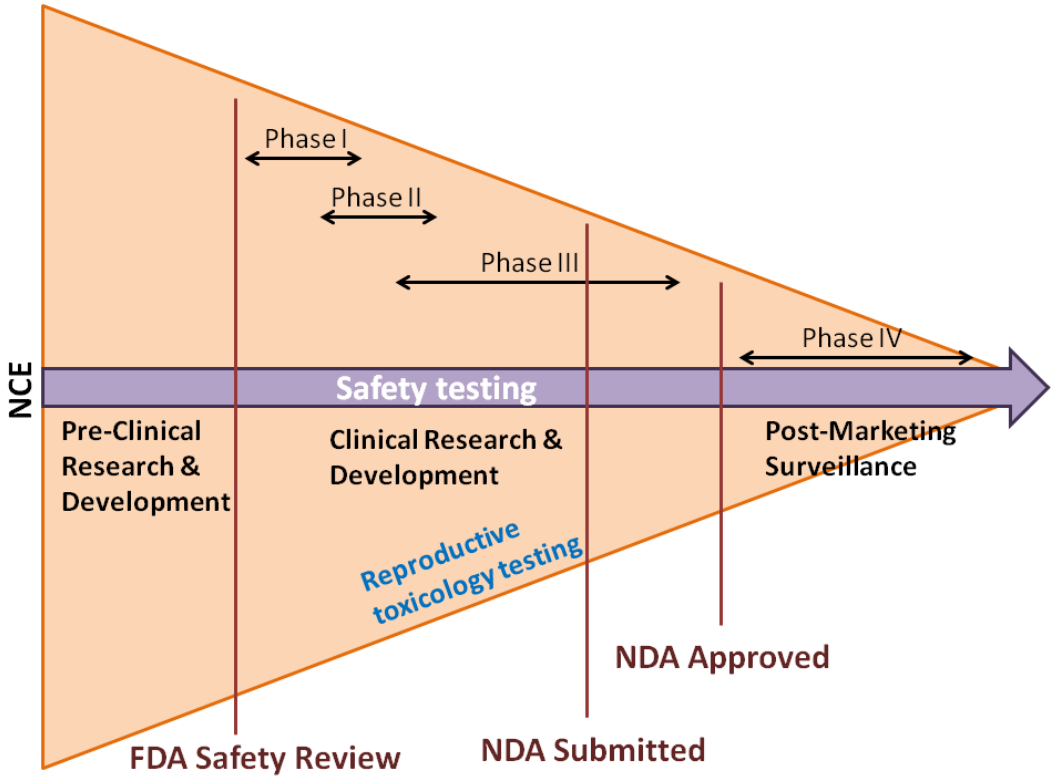


Figure 3.1. Different phases of pharmaceutical drug development (USA) (**adapted from Cortvrindt and Smitz, 2002).** Safety testing is a continuous process that starts during the pre-clinical phase and ends with post-marketing surveillance. Reproductive toxicity tests are carried out during the clinical test phase.

3.1.1 Regulatory toxicity testing of pharmaceuticals

Most regulatory testing requirements within the pharmaceutical industry are controlled by the EMA (European Medical Agency), FDA (USA) and PMDA (Pharmaceuticals and Medical Devices Agency, Japan). The guidelines are laid out in the core tripartite harmonised guideline issued by the International Conference on Harmonised tripartite guideline for the detection of toxicity to reproduction for medicinal products and toxicity to male fertility:ICH S5 (R2) 1993 which provides guidance on tests for reproductive toxicity (ICH, 1993) (2000 addendum). In addition, a second ICH M3 (R2) guideline for the non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals is also consulted.

The ICH (R2) guideline contains a description of the testing concept and recommendations, especially those addressing pre-mating treatment duration and suggested observations to assess for reproductive toxicity (ICH, 1993) (2000 addendum). The ICH guidelines define the periods of treatment to be used in animals to assess for reproductive risk: fertility, implantation through organogenesis to closure of the hard palate and the pre and post-natal period through to the end of lactation. This allows identification of specific toxicity at key stages of the reproductive cycle.

3.1.2 Regulatory toxicity testing of agrochemicals

Agrochemicals, such as crop protecting agents, wood preservatives and veterinary disinfectants can result in potential health risks for anyone exposed to such chemicals. The preclinical designs to assess effects of potential toxicants on the pre-natal ovary however, differ somewhat between pharmaceutical compounds (see previous section) and agrochemicals. While pharmaceutical companies analyse effects on long-term outcome, such as pregnancy after exposure *in utero,* agrochemical testing also includes a more detailed qualitative and quantitative histological assessment of the primordial follicle pool following pre-natal exposure. This involves dosing female rats 2 weeks prior to ovulation, to examine immediate effects on ovulation and fertilisation. Although the fundamental study designs for agrochemicals and pharmaceuticals are fairly similar, studies still have to use different approaches, partly due to the fact that therapeutic levels of pharmaceutical exposure is well studied, with controlled therapeutic exposure, whereas environmental agrochemical exposure is more difficult to predict, given that we do not know their levels in the environment or the likely duration of their exposure. Furthermore, the type of reproductive toxicology studies used in academic research also differ considerably from regulatory research, with academic testing usually focusing more on mechanistic studies, whereas regulatory research tends to concentrate on assessing potential risk using qualitative morphological examination of the cellular components of the ovary alongside examining effects on fertility.

3.1.3 DNA gyrase inhibitors for antibiotic development

In recent years, inhibitors of Type II topoisomerases such as DNA gyrases and topoisomerase IV have become widely used as targets by the pharmaceutical industry in the development of novel antibiotics (Alt et al., 2011). Since topoisomerases are essential for cell function (Section 1.4) they have become particularly attractive targets in the development of novel antibiotics, as bacterial topoisomerases can be inhibited and bacterial function repressed. This is particularly important in the fact of today's ever-increasing threat of bacterial antibiotic resistance, in particular pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Tse-Dinh, 2007, Oblak et al., 2007, Srommenger et al., 2014).

Preliminary investigation into the expression pattern of Topo IIα in the developing rat ovary demonstrated that its expression was confined to the germ cells in the pre-natal ovary, but became localised to the surrounding granulosa and stromal cells a few days after birth, around the same time as follicles began to form (personal communication, Dr. N Powles-Glover, AstraZeneca).

3.1.3.1 AZTC

Various novel antibiotics have been under pharmaceutical development at AstraZeneca. These antibiotics include bacterial DNA gyrase (Topo II) inhibitors, many of which have known homologues in mammalian cells. Some Topo II inhibitors affect spermatogenesis in a manner that indicates an effect on meiosis (Kallio and Lähdetie, 1997, Russell et al., 1998, Russell et al., 2000). The work presented here outlines the *in vivo* effects of a Topo II inhibitor: AZTC (Section 1.4.2.1). This compound was selected due to preliminary *in vivo* toxicity studies demonstrating testicular effects.

The *in vivo* AZTC study was planned by AstraZeneca independently of this PhD studentship, with the initial *in vivo* studies carried out shortly after the start of my PhD (Fig. 3.2). AZTC was selected as a potentially good compound to use to compare *in vitro* studies with the *in vivo* ones that had already been carried out at AstraZeneca. I therefore obtained and analysed ovaries from PND5, PND15 and

adult (13+ week) females exposed to AZTC *in utero,* using material that was supplementary to AstraZeneca's *in vivo* safety assessment studies.

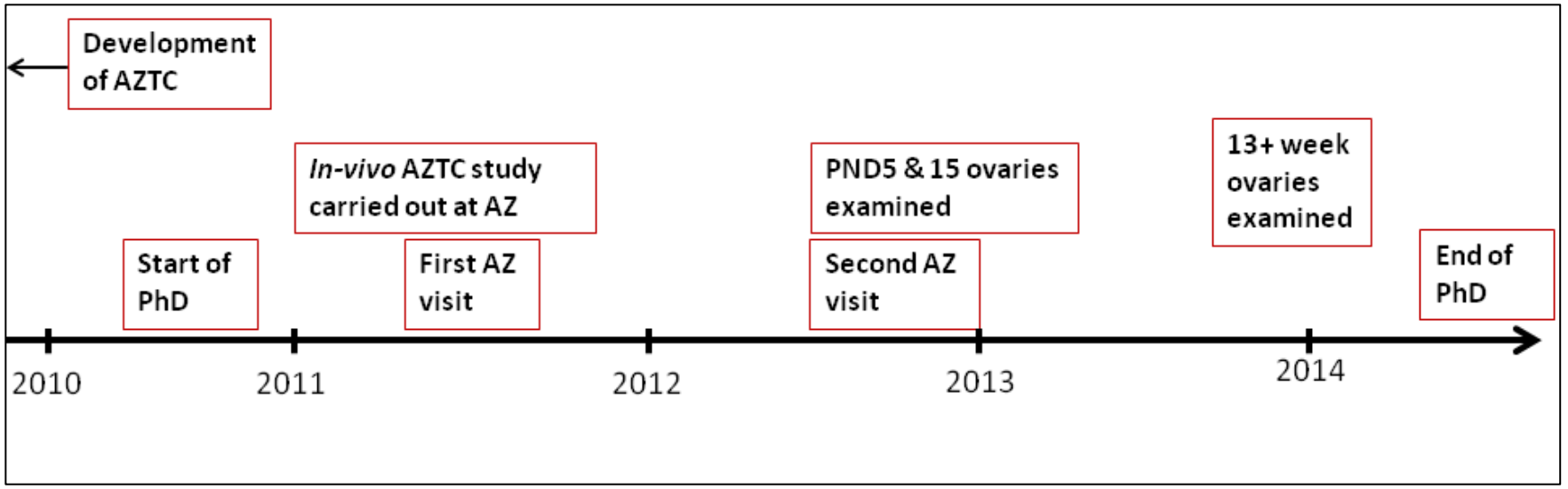


Figure. 3.2. A timeline of the *in vivo* AZTC study from the start (Sept 2010) to completion (Aug 2014) of the PhD. The initial safety study of AZTC was carried out in 2011, where female rats were dosed with AZTC and ovaries from PND5 and PND15 and adult F1 females were collected. The first visit to AZ was carried out during this time. The second AZ visit was in 2012 when ovaries from PND 5 and PND15 F1 females were cut, stained and analysed. The follicles from these ovaries were counted and categorised. The final 13+ week F1 ovaries were cut, stained and analysed in Edinburgh.

3.2 Aims

The aim of this work was to analyse the size and health of the ovarian follicle pool in female rats that had been exposed to AZTC *in utero*. This was done to investigate how AZTC might affect the developing ovaries, since exposure occurred during the time period covering PGC proliferation, germ cell nest breakdown and meiosis.

3.3 Methods

3.3.1 Study design

All the *in vivo* work described here was carried out by AstraZeneca staff on site, prior to and during my first visit to AstraZeneca. The studies were designed by Dr. N Powles-Glover and Dr. Jane Stewart. The oestrous cycles of female Han Wistar rats were monitored for 14 consecutive days, prior to mating with proven males. They were paired 1:1 with unrelated males of the same strain. Day 0 post-coitum was selected when the first evidence of mating was observed, identified by a vaginal plug *in situ* or by the presence of sperm in a vaginal smear. This was also the first day of dosing for females. The males were only used for mating and were not dosed.

Females were dosed either with the vehicle control or one of two selected concentrations of compound from Day 0 post-coitum until parturition, therefore covering the period from implantation, embryonic and fetal development, up-to parturition. The high dose had previously reported effects on spermatogenesis in male rats (personal communication, Dr. N Powles-Glover, AstraZeneca). The lower dose was selected to evaluate a dose response. The test formulation was made up in vehicle (consisting of water containing 0.5% w/v hydroxypropyl methylcellulose and 0.1% w/v polysorbate 80). The dose volume was 10 ml/kg and individual dose volumes were based on recorded body weight of each animal. Females were dosed once every morning, by oral administration (gavage) (Appendix B, pg. 260).

All females were dosed from the day of mating until parturition and were allowed to litter. Females received either the control vehicle, low dose or high dose. F1 female pups were collected on days 5 PP (only control and high dose) and 15 PP (control, low and high dose). The gonads were dissected from the body and mounted

individually in 3% agarose gels and immersed in fixative (10% buffered formalin). The remainder of the F1 generation were weaned and allowed to mature. Oestrus cycle monitoring was carried out on the females from the F1 generation from day 64 post-partum. A proportion of the control F1 males were retained until sexually mature, and were subsequently mated with treated F1 females. The remaining sexually mature F1 males were euthanised. Finally, ovaries from 13+ week old un-mated F1 females were also collected and fixed as above (control, low and high doses). Ovaries were processed and embedded in wax by AstraZeneca staff as outlined in Section 2.2.

3.3.2 Histological assessment

Following the *in vivo* study outlined above which was conducted by AstraZeneca staff, I carried out the histological analysis of the PND 5, 15 and 13+ ovaries. Ovaries to be analysed were sectioned at 5 μm and H&E stained (Sections 2.2.3-2.2.4). Ovaries from PND 5, 15 and 13+ week old females were examined, with every 6th section from PND 5 ovaries, every 20th section from PND 15 ovaries and every 50th section from 13+ week old F1 females. Follicle numbers, distribution and health was analysed in PND 5 and 15 ovaries. The 13+ week ovaries were analysed only to ascertain primordial follicle numbers and health. Due to effects observed primarily in primordial follicles in PND 15 ovaries it was considered not possible to successfully analyse other follicle types at this age. Due to the large volume of the ovary and the numerous CL, counting other follicle types would have been difficult to do reliably and efficiently. All follicle analysis was conducted blind to treatment.

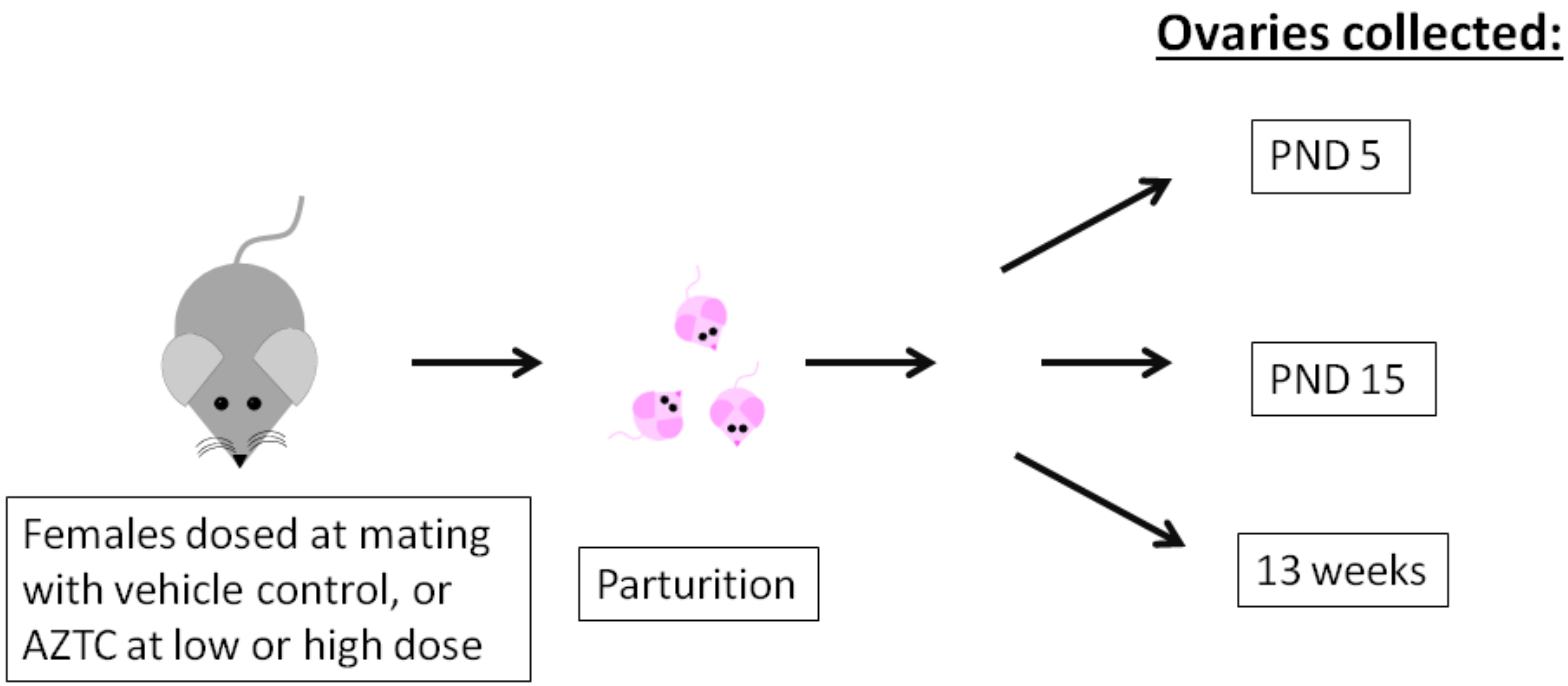


Figure 3.3. Study design for pre-natal *in vivo* exposure of AZTC. Pregnant females were dosed by oral administration with vehicle control, or compound at either a low or high concentration, once daily until parturition. Ovaries from F1 females were collected at PND 5, PND 15 or at 13 weeks.

	Treatment	Mother #	Pup #
PND 5	Control	22	4
		23	1, 3
		28	2, 3
	High dose	45	2, 3, 5
		48	1, 2
PND 15	Control	1	1
		2	2, 4
		5	3, 4
		6	1
	Low dose	8	1, 2
		9	1, 2, 3
	High dose	14	1
		16	2, 3
		17	2, 3, 4, 5, 6
13+ weeks	Control		343, 344, 347, 349
	Low dose		366, 371, 375
	High dose		89, 90, 91, 92

Table 3.1. Study design outlining pup numbers from which ovaries were collected, cut and analysed, relative to mothers and dosage groups. Only one ovary was collected for histological analysis from each pup. At PND 5, a total of 5 pups were collected from 3 different mothers treated with control vehicle and 5 pups were collected from 2 mothers that had received treatment. At PND 15, 6 pups were collected from 4 different control mothers, 5 pups were collected from 2 mothers that had received the low dose, and 5 pups were collected from only one mother that had received the high dose of AZTC.

3.3.3 Statistical analysis

Graphpad Prism was used for all statistical analysis of follicle number and distribution between control and treated ovaries. For all studies involving more than one dose group, data normality was assessed using Kolmogorov Smirnoff tests. Where data was not normally distributed, the Kruskal-Wallis non-parametric test was used to analyse the data. This was followed by a Dunns post-hoc test if the Kruskal-Wallis test showed a significant difference. Where the data was normally distributed, a one-way ANOVA was used to determine if there were any significant differences between treated and untreated ovaries. This was followed by the Bonferroni post-hoc test if the ANOVA showed a significant difference. Since the PND 5 study only had two treatment groups, a two-tailed unpaired t-test was carried out on the data obtained from follicle counts on PND 5 ovaries.

3.4 Results

3.4.1 PND 5 ovary analysis

Ovaries from PND5 females exposed to vehicle control or the high dose of AZTC *in utero* were collected and every 20th section was analysed for follicle numbers, distribution and health (Sections 2.2.3, 2.2.4 and 2.3). Unfortunately, the majority of the ovary sections had histological problems whereby many of the ovaries (both control and treated) had poor follicle morphology and appeared striated. It was therefore difficult to assess health properly in these sections, as it was not possible to be certain whether some follicles were unhealthy or whether they had an abnormal appearance due to poor histology (Fig. 3.4).

No effect of AZTC was observed on follicle numbers (Fig. 3.5i), distribution (Fig. 3.5ii) or health (Fig 3.6i,ii) in ovaries from exposed females compared with *in vivo* (n=5 for both groups). Despite the poor histology, though, there did appear to be a slight, non-significant decrease in the number of unhealthy follicles in treated PND5 ovaries (Fig. 3.6) (p=0.205).

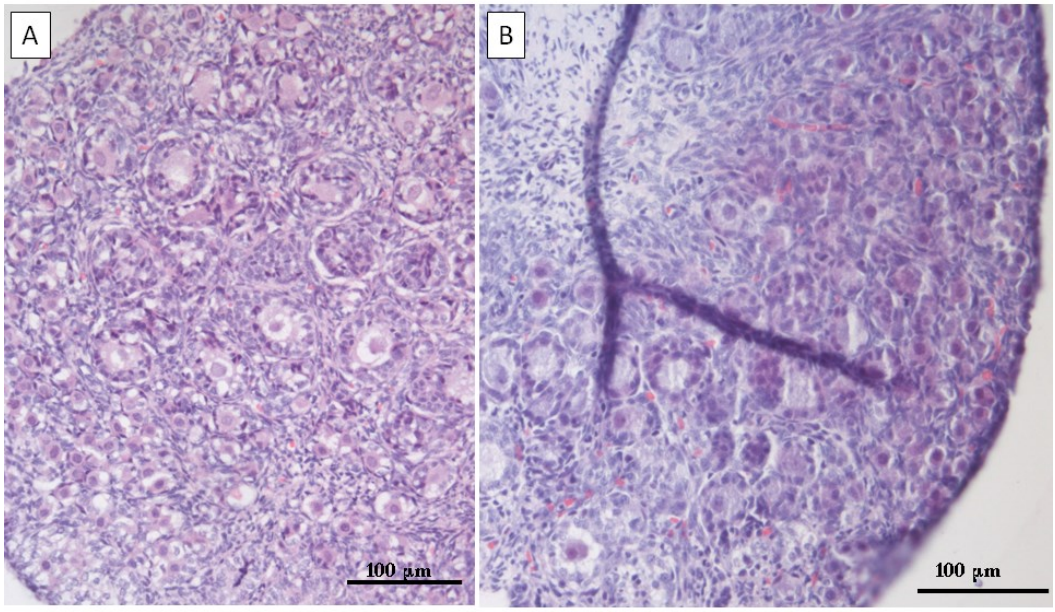
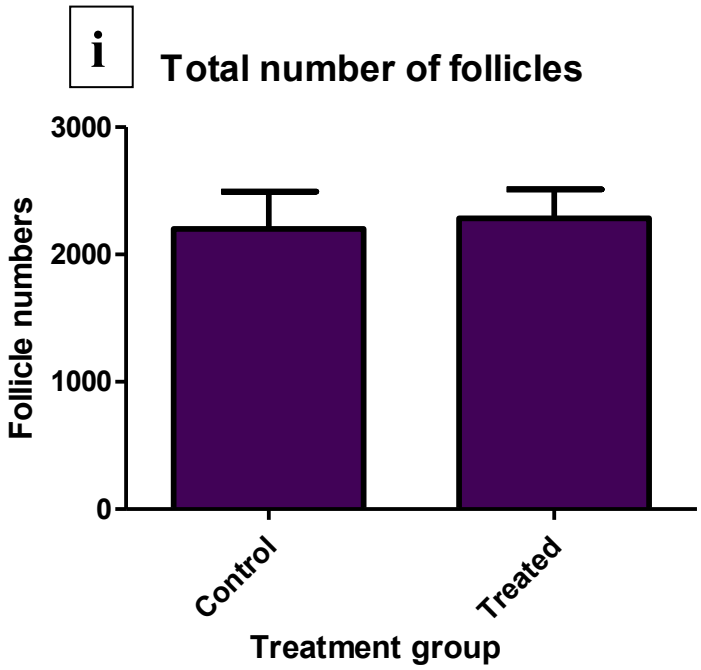


Figure 3.4. Representative photomicrographs of PND5 ovaries from control (A) and treated (B) females, where poor histological morphology made follicle counting/assessment difficult (in comparison with other ovaries from the same age of rats).



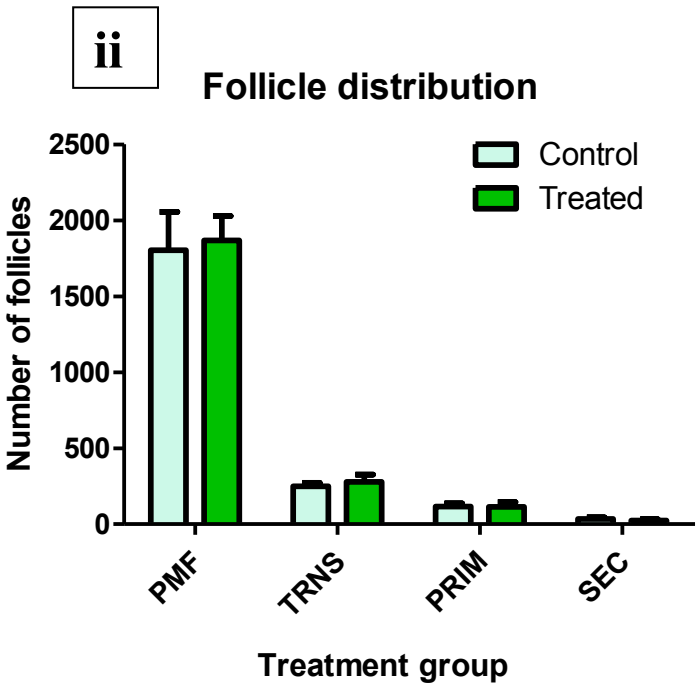


Figure 3.5. Total number (i) & distribution (ii) of follicles in PND5 ovaries following exposure to AZTC *in utero.* No effect on follicle numbers was observed in AZTC exposed PND 5 ovaries when compared with control ovaries (p=0.8271) (i). Similarly, no effect was observed on follicle distribution within exposed ovaries (p=0.9735) (ii). Bars denote mean + sem; n=5 for both groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary

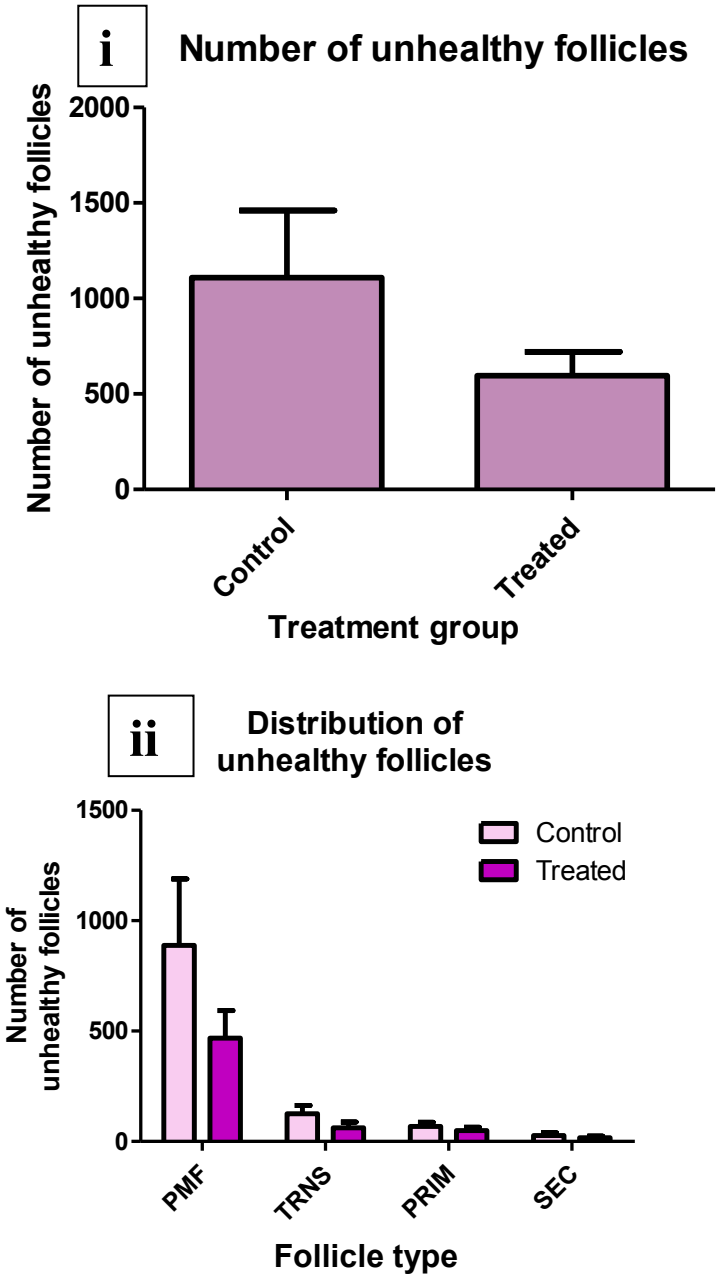


Figure 3.6. Distribution of unhealthy follicles within ovaries of AZTC treated PND5 females. AZTC exposure had no significant effect on the number of unhealthy follicles within PND 5 ovaries, when compared with control ovaries (p=0.205) (i). Similarly, no significant difference was observed in follicle distributions between control and treated ovaries although there appeared to be a slight non-significant decrease in the number of unhealthy primordial (p=0.232) and transitional follicles (p=0.206) in exposed ovaries (ii). Bars denote mean + sem; n=5 for both groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary.

3.4.2 PND15 ovary analysis

Ovaries from PND15 females exposed to low or high dose of AZTC *in utero* were collected and every 40th section was analysed. Follicles were counted, and follicle distribution and health was analysed (n=5 for all groups).

There were pups born to two high dose mothers (#14 & 16, see Appendix B) that appeared to have severely affected ovaries (Fig. 3.7). However, due to uncertainty over their exact provenance, they were excluded from the study. I was restricted to the use of ovarian tissue provided by AstraZeneca, and as a result, only the ovaries from female pups born to the high dose mother #17 could be analysed for the high dose group.

Pre-natal exposure of females to the high dose of AZTC resulted in a significant increase in the number of follicles per ovary when compared with low-dose ovaries and controls (Fig. 3.8). When the follicles were classified into their follicle types it became clear that rise in follicle number was due to an increased number of primordial follicles in pup ovaries in the high-dose group (Fig 3.9i). A significant increase in the number of unhealthy follicles in ovaries of females exposed to the high dose of AZTC was also observed (Fig. 3.10i). A dose-dependent non-significant trend was also observed with increasing number of healthy follicles within exposed ovaries with increasing AZTC concentration (Fig. 3.10ii). When the unhealthy follicles were classified into their follicle types a dose dependent increase in unhealthy primordial and transitional follicles was observed, reaching significance at the high dose when compared with controls and low-dose ovaries (Fig. 3.11i). A similar trend was observed when the percentage of unhealthy primordial follicles was considered, although this was not significant (Fig. 3.11ii).

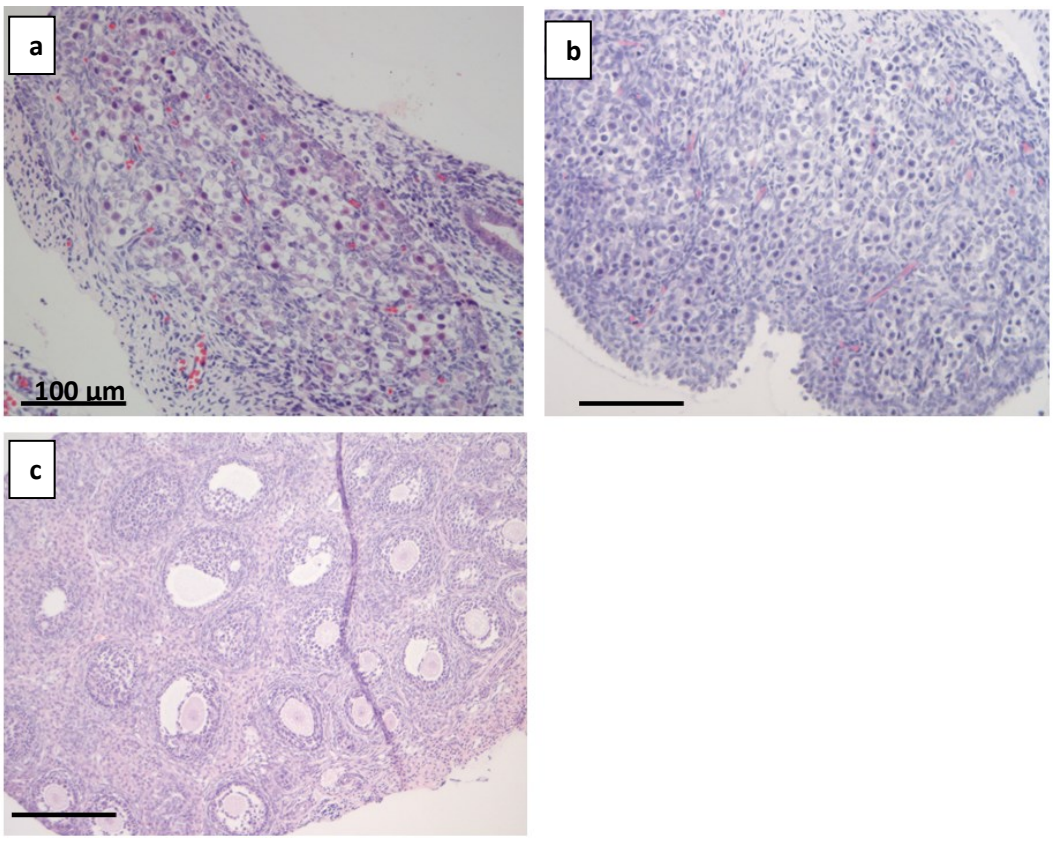


Fig 3.7. Representative photomicrographs of ovaries from females pups born to mothers 14 and 16. Ovary sections from PND 15 females born to females 14 (a), 16 (b) and 17 (c). Ovaries from females born to females 14 and 16 lacked follicles and were therefore not included in the analysis.

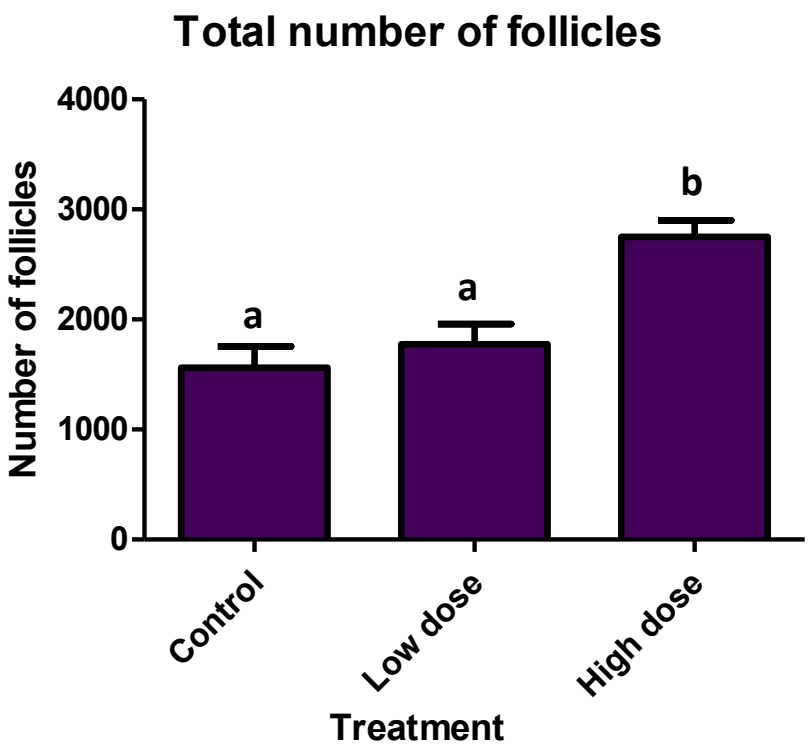


Figure 3.8. Mean follicle numbers in PND 15 ovaries from treated and control females. A significant increase in follicle numbers was observed in ovaries exposed to the high dose of AZTC when compared with control and low dose ovaries ($p=0.0002$). Bars denote mean + sem; n=6 for controls and n=5 for both treatment groups. Means with different letters are significantly different ($p<0.05$).

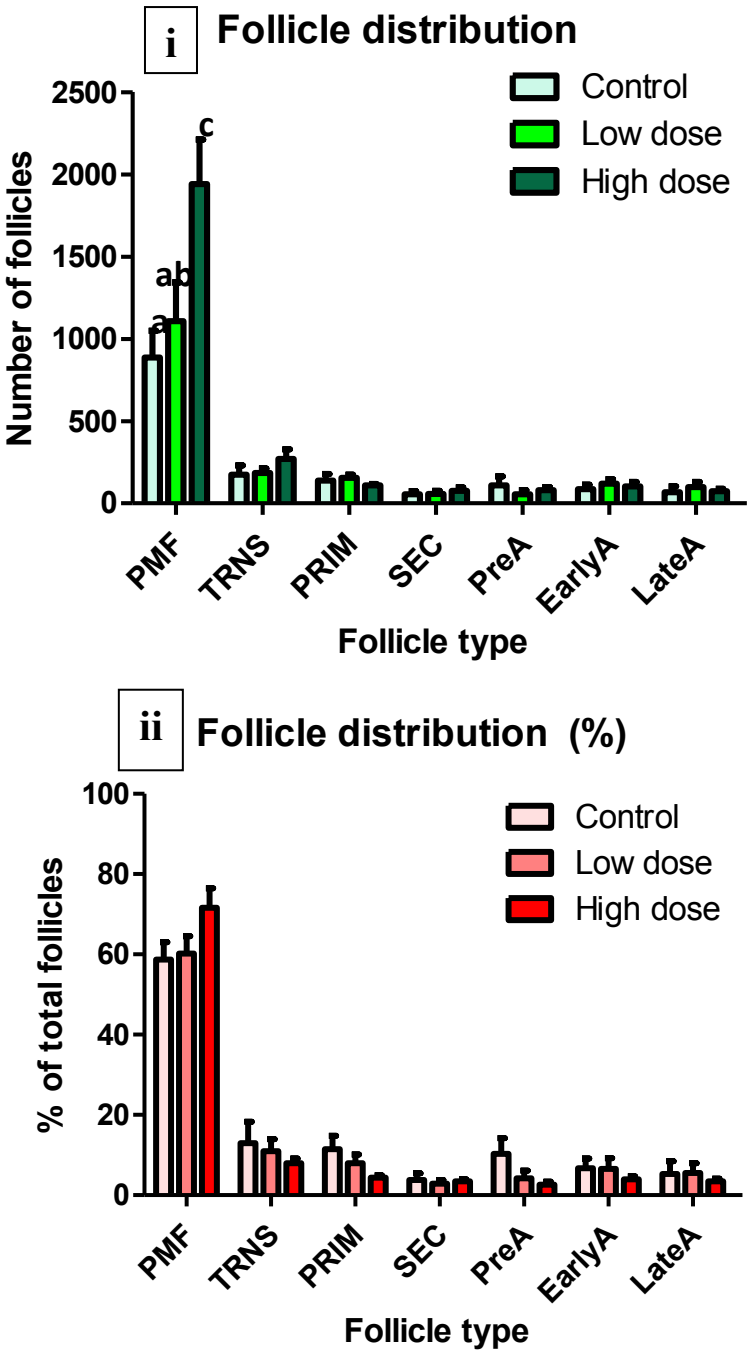


Figure 3.9. Comparison of follicle distribution between control and treatment PND 15 ovaries, presented as total numbers (i) and percentages of total follicles (ii). There was a significant increase in the number of primordial follicles in ovaries of females exposed to the high dose of AZTC ($p=0.016$) (i). There was no significant effect on the number of other follicle types in the exposed ovaries. No significant difference was observed in percentages of follicle types across treatment groups (ii). Bars denote mean + sem; n=6 for controls and n=5 for both treatment groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary, PreA: Pre-Antral, EarlyA: Early-Antral, LateA: Late-Antral. Means with different letters are significantly different ($p<0.05$).

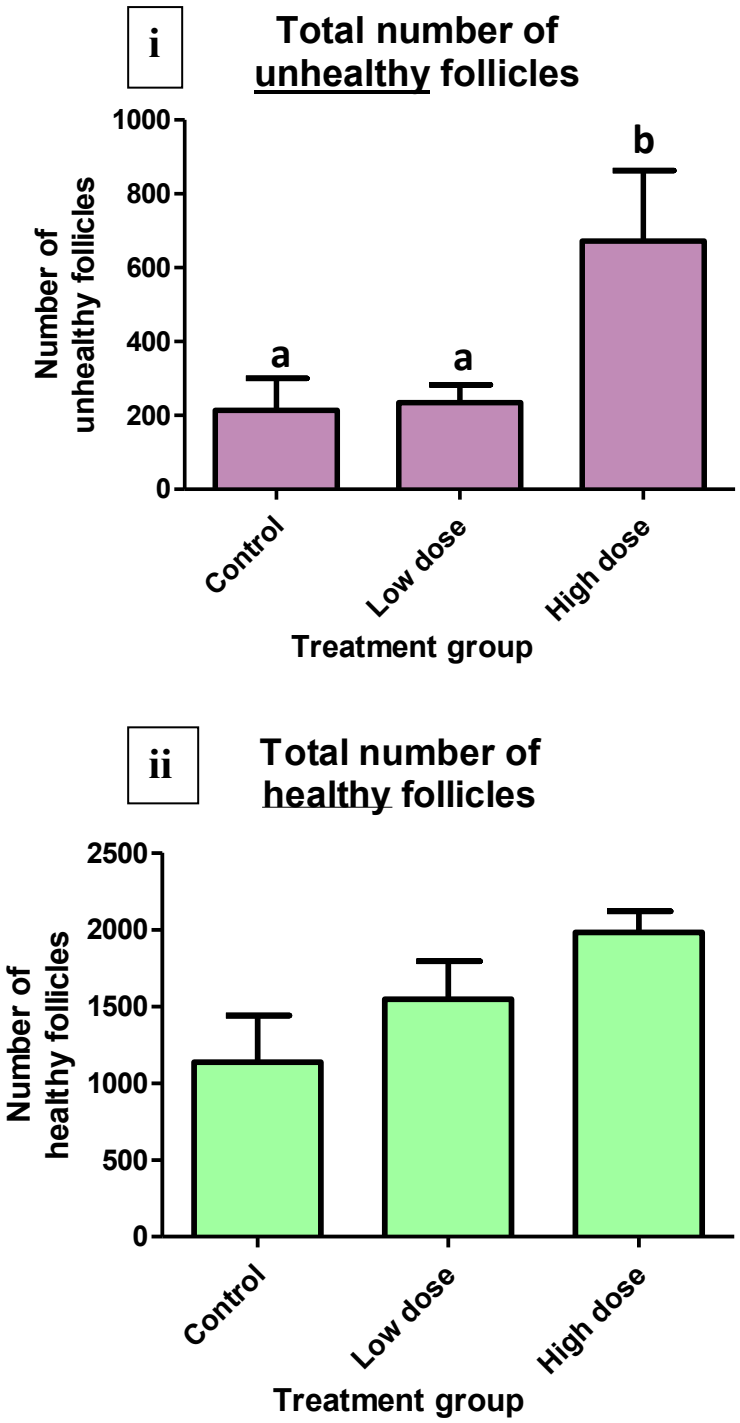


Figure 3.10. Total number of unhealthy (i) and healthy (ii) follicles within ovaries of PND15 females. Ovaries exposed to the high dose of AZTC had significantly more unhealthy follicles than did low dose and control ovaries (p=0.038) (i). A non-significant dose dependent trend was also observed, with a slight increase in the number of total healthy follicles observed with increasing AZTC dose (p=0.091) (ii). Bars denote mean + sem; n=5 for all groups. Means with different letters are significantly different (p<0.05).

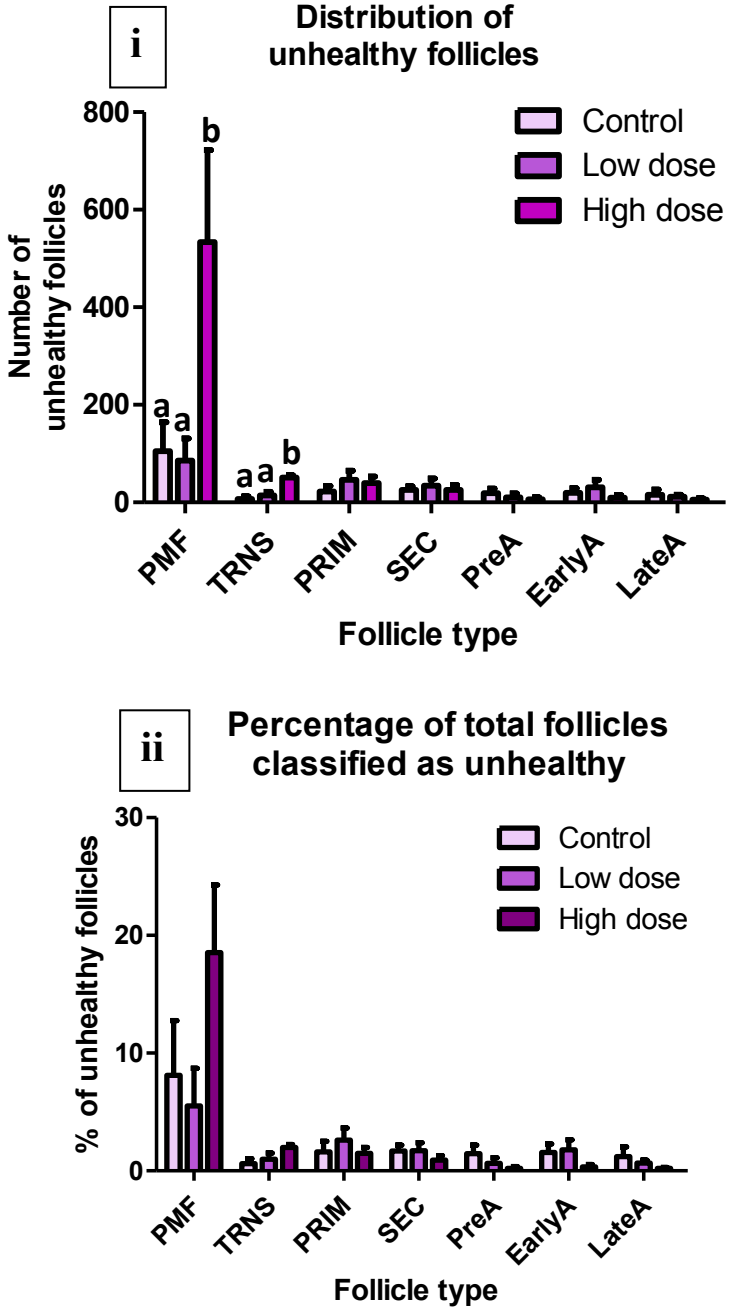


Figure 3.11. Comparison of the distribution of unhealthy follicles between control and treatment PND 15 ovaries, presented as total numbers (i) and percentages of total follicles (ii). Ovaries exposed to the high dose of AZTC had a significantly higher number of unhealthy primordial ($p=0.032$) and transitional ($p=0.0078$) follicles than did control and low dose ovaries. No significant differences were observed in the percentages of unhealthy follicles across treatment groups. Bars denote mean + sem; n=6 for controls and n=5 for both treatment groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary, PreA/EarlyA/LateA: Pre-, Early- and Late Antral. Means with different letters are significantly different ($p<0.05$).

3.4.3 Primordial follicle numbers in AZTC exposed 13+ week old ovaries

To investigate if the observed increase in total follicle numbers and unhealthy follicles in AZTC-exposed PND15 ovaries was still observed when the females reached adulthood, ovaries from 13+ week old females that had also been exposed *in utero* were analysed histologically for primordial follicle numbers and health. Every 50th section was H&E stained and PMFs within these sections were counted and classified according to health. In this case, only PMFs were analysed, with all other follicle types excluded from the analysis. No significant effects of AZTC were observed on total number of PMFs or on their health in 13+ week ovaries (Figs 3.12-3.13).

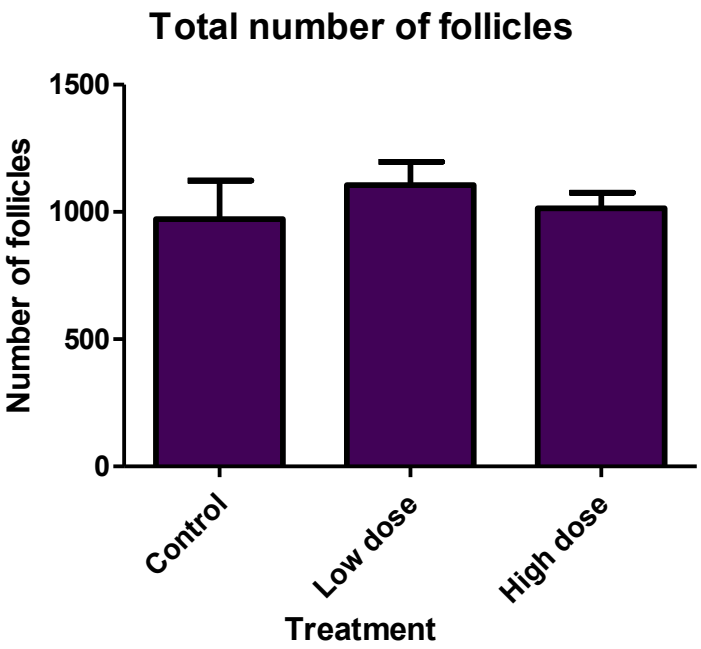


Figure 3.12. Total number of PMFs in ovaries 13+ week old females exposed to AZTC *in utero.* No significant difference was observed between PMF numbers in control, low- or high-dose ovaries (p=0.725). Bars denote mean + sem; n=6 for controls and n=5 for both treatment groups.

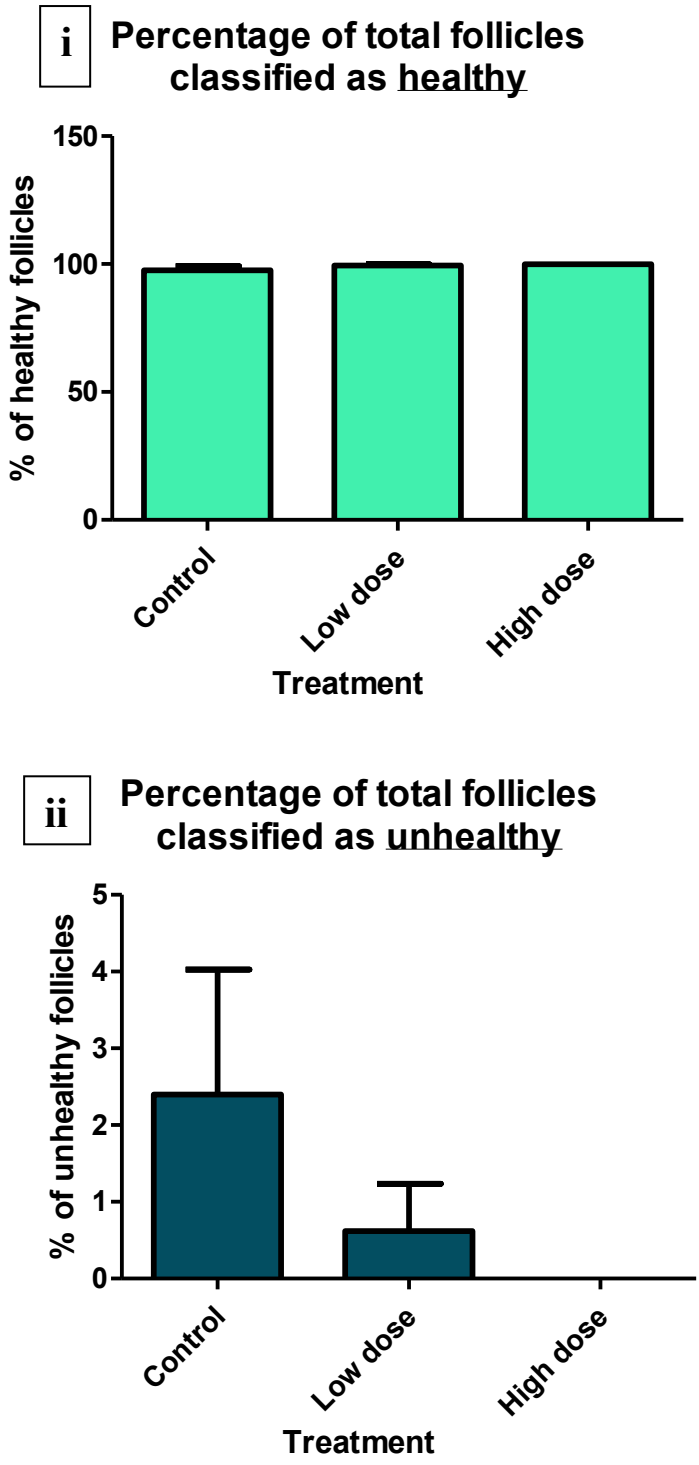


Figure 3.13. Percentage of healthy (i) and unhealthy (ii) PMFs in ovaries 13+ week old females exposed to AZTC *in utero.* No difference in percentage of healthy or unhealthy (p=0.293) PMFs was observed between treatment groups. Bars denote mean + sem; n=4 for control and high dose groups, n=3 for low dose group.

3.5 Discussion

In this study, female rats were mated and exposed to AZTC daily from the day of conception until parturition. Ovaries from F1 female pups that had been exposed to AZTC *in utero* were collected at varying time points: PND 5, PND 15 and 13+ weeks pp.

3.5.1 Effect of pre-natal *in vivo* AZTC exposure on ovarian follicles

Histological analysis of the ovaries revealed that no effect of AZTC exposure was observed within ovaries at PND 5. By PND 15 however, ovaries exposed to the high dose of AZTC had increased follicle numbers when compared with low dose and control ovaries. When the follicles were classified into their follicle types it became clear that the rise in follicle number was due to an increased number of primordial follicles in ovaries of the high-dose group. This was a surprising result as inhibition of mammalian Topo II, if it is indeed occurring here, would be expected to inhibit proliferation of cells within the ovary, resulting in a reduction of follicle number, rather than an increase. The lack of any effect of AZTC within the 13+ week old ovaries suggests that either the effect of AZTC on PMF numbers did not persist and was corrected in later life, or that the effect might have been there, but too small to identify. On the other hand, the possibility of the PND15 finding being due to a litter effect must also be considered, since all the female PND 15 pups in the high dose group came from the same female. This was clearly far from ideal, where a minimum of 3-4 females should be used to avoid the possibility of a potential litter effect.

In the PND 5 ovaries, follicle health was difficult to analyse robustly due to poor histological preparation of the tissue. None-the-less, there was no evidence of PND 5 ovaries being affected by AZTC exposure. Exposed ovaries did, in fact, appear slightly healthier than controls, but this was not significant. When the health of the PND 15 ovaries was analysed however, there was a significant increase in unhealthy follicles, in particular primordial and transitional follicles, within the ovaries exposed to the high dose of AZTC. It was noted that although a large proportion of the 'surplus' follicles were unhealthy, there was also a slight but non-significant increase in the number of healthy follicles. The unhealthy follicles would have presumably

gone on to become atretic and die, possibly explaining the absence of an effect of treatment on follicle numbers in the adult ovaries collected at 13+ weeks. The PND 15 effect on ovary health could, however, also be a litter effect.

Germ cell nest breakdown, which occurs during pre-natal ovary development, is usually accompanied by a large wave of oocyte atresia. It is possible that AZTC interfered with germ cell nest breakdown and resulted in the higher number of surviving oocytes observed here. Previous studies investigating the effects of compounds interfering with germ cell nest breakdown have frequently reported the presence of binuclear oocytes or MOFs within treated ovaries, which are therefore considered an expected result of disruption to germ cell nest breakdown (Kim *et al.,* 2009), although no such binuclear oocytes or MOFs were observed here. This suggests that AZTC might not have been interfering in germ cell nest breakdown, or if it was, might have been doing so by an alternate pathway. A more likely possibility is that AZTC inhibited apoptosis and resulted in more surviving oocytes forming follicles. Germ cell death during nest breakdown has been suggested to be a 'quality control' mechanism, where abnormal or lower quality germ cells are discarded. It is therefore possible that if some germ cells 'escaped' the wave of apoptosis due to the presence of AZTC, they ended up forming follicles of lesser quality, thereby becoming unhealthy.

A possible explanation for the lack of observed effects of AZTC at PND 5 is that they might not have been apparent yet at this point. If AZTC did have an effect on germ cell development, it may not have become apparent until a week or two after birth. The PND 5 ovaries might also not have been counted as effectively as the PND 15 ovaries, as many were striated and difficult to analyse. It is possible that the effect observed at PND 15 may have corrected itself by adulthood, with the unhealthy follicles becoming atretic and dying, consequently reducing the number of follicles in the ovary to a similar number to that observed in the control ovaries by adulthood. As outlined above, however, it should be borne in mind that all high dose female offspring come from the one mother. Given that, the effect of AZTC here could, instead be a result of abnormal development in one female's litter.

Immunohistochemical analysis of ovary health would have been ideal within these ovaries to identify apoptotic cells within exposed ovary, as well as to further support the results obtained by histological examination. Immunohistochemical staining for Cleaved-Caspase 3 was attempted (Cell Signalling, 9661) but this proved unsuccessful with no staining showing up in the tissue, possibly due to the poor morphology, over-staining or due to the antibody. This was not taken further due to the worries that the tissue was simply not high enough quality for immunohistochemistry.

AstraZeneca had also collected ovaries from earlier time-points, during embryonic development and at birth. Time allowing, it would have been interesting to investigate the observed effect on follicle numbers using immunohistochemistry for a germ cell marker such as VASA, Gdf9, Zp3 and Figla, or Figla, (Hu et al., 2012) on embryonic ovaries to examine germ cell numbers within the embryonic ovaries during AZTC exposure, prior to follicle formation.

Finally, it would have been ideal to investigate the observed effects in the PND 15 study further by repeating the study with more litters being born to different exposed mothers. Unfortunately, this was not possible.

3.5.2 Mechanisms of action of AZTC

AZTC inhibits bacterial topoisomerase, which is believed to have mammalian homologues. Topoisomerases serve various functions in mammalian cells, being involved in both mitotic and meiotic divisions. As outlined above (Section 3.5.1), it is possible that AZTC was likely to inhibit mammalian topoisomerase, and therefore the expected outcome had been a reduction in oocyte numbers due to a potential effect on PGC proliferation or meiosis. No direct effect on somatic cells was expected as the exposure window in this study coincided with the period when Topo II is expressed in germ cells only. It was believed that if Topo II was inhibited within the ovarian germ cells, mitotic divisions were likely to become altered during PGC proliferation and fewer of the germ cells invading the ovaries during embryonic

development would be capable of dividing and increase germ cell number in the ovaries. Surprisingly, the opposite effect was observed here, with AZTC instead resulting in an increase in primordial follicle numbers. It is therefore possible that AZTC might not be inhibiting Topo II in mammalian cells, but is instead having a secondary or off-target effect resulting in the observed increase in primordial follicle numbers, although this would require further investigation.

Unfortunately, the effects of AZTC on meiosis *in vivo* could not be determined. From histological analysis, the large majority of oocytes appeared to be in dictyate, but in order to fully investigate if AZTC was affecting meiosis in the pre-natal ovary, other tests would need to be carried out. Chromosome spreads on oocytes following pre-natal *in vivo* exposure, where the synaptonemal complex and pairing of the homologus chromosomes is visualised would be an interesting study to carry out. Other methods that could have potentially been carried out also involve immunhistochemistry to visualise meiotic markers such as Sycp3, Stra8 (Hu et al., 2012) or diplotene markers Msy2 (Gu et al., 1998), PAR6 (Wen et al., 2009) or TRP63 (Suh et al., 2006, Myers et al., 2014).

3.6 Conclusions

An increase in follicle numbers and unhealthy follicles was observed within ovaries of 15 day old females exposed pre-natally to the high dose of AZTC *in vivo*. This was not consistent with effects observed in exposed ovaries when collected at earlier (PND5) or later (13+ weeks) time points. It is possible that the effect is solely due to a litter-effect in the one mother that gave rise to all female whose ovaries were examined at PND15. The results observed were also inconsistent with the expected effects of Topo II inhibition, indicating that AZTC might be having a secondary or an off-target effect resulting in these observed results.

Chapter 4:
The development of an embryonic mouse ovary culture system

4.1 Introduction

The pre-natal period of mammalian ovary development is a sensitive, yet crucial one, as it is during embryonic development that primordial germ cells migrate to the developing ovary, undergo a phase of rapid cell proliferation, enter prophase I of meiosis up-to dictyate, followed by a breakdown of germ cell nests and in some species, primordial follicle formation. The size of the primordial follicle pool in the newborn ovary determines, in part, the female's reproductive lifespan (Schindler et al., 2010). Follicle development is dependent on the establishment of an appropriate communication pathway between the oocyte and the surrounding granulosa cells through gap junctions, tranzonal projections, as well as via paracrine and endocrine signals (Sections 1.1.1.4 & 1.1.2.2). Despite this, relatively little is known about the factors and mechanisms that regulate early germ cell development, the establishment of the primordial follicle pool and the initiation of follicle growth.

Various culture techniques have been developed to investigate early gonadal development in mouse (Adams and McLaren, 2002, Zhang et al., 2012, Buehr et al., 1993), hamster (Yu and Roy, 1999) and in the human (Brieno-Enriquez et al., 2010, Childs et al., 2010, Angenard et al., 2011a, Farhi et al., 2011, Hartshorne et al., 1999). Ovary culture can be a useful tool to investigate not only the factors and processes of early ovary development, but also the effects of potential reproductive toxicants on the ovary. However, to the best of my knowledge, the large majority of reproductive toxicity studies that have been carried out *in vitro* have examined effects on the post-natal ovary. This is possibly due to the fact that post-natal ovary and pre-antral follicle culture systems are more widely used and are fairly robust, but it also highlights the lack of an adequate embryonic ovary culture system for use in reproductive toxicology.

Within the pharmaceutical industry, the majority of pre-clinical reproductive toxicity studies carried out to investigate the effects of pharmaceutical compounds on the pre-natal ovary consist of assessing fetuses exposed to a compound *in utero.* Although this detects any immediate drastic reproductive effects associated with dysgenesis of the reproductive system, such as underdeveloped or dysfunctional ovaries and/or

reproductive tract, it does not pick up on subtle effects that the compound may have on the size of primordial follicle pool. Any compound that reduces the size of the primordial follicle pool could potentially shorten the reproductive life-span of the female. However, this would not be detected until much later, after the majority of time-points usually examined within the pharmaceutical industry. Since a woman's reproductive life-span is considerably longer than that of a rodent, it is crucial that a compound which can result in a premature exhaustion of the primordial follicle pool is discovered, as this could result in premature ovarian insufficiency (POI), defined by amenorrhea before the age of 40 (De Vos et al., 2010). The ability to demonstrate an effect of a compound on the pre-natal ovary, and compare these with effects to the neonatal or adult ovary, is therefore of novel and general interest to the reproductive toxicology community.

4.1.1. Current available embryonic ovary culture systems

The majority of available culture systems involve the post-natal ovary and germ cells that have already entered meiotic arrest. The fetal stage of reproductive development is a crucial and sensitive period, as it includes the migration of germline stem cells, and their proliferation, as well as entry into the first meiotic division. Pre-meiotic germ cells, not enclosed in follicles, may well be directly responsive to chemicals, making fetal development a particularly vulnerable period (Anderson et al., 2014). A culture system whereby embryonic mouse ovaries, containing oogonia undergoing the first meiotic division, can be cultured intact would have the potential to provide an important contribution to the field of reproductive biology and toxicology, particularly as aneuploidy predominantly occurs during the first meiotic division of oocytes (Eichenlaub-Ritter, 1996, Eichenlaub-Ritter, 1998, Plachot, 2001).

As outlined previously (Section 1.3.1), any factors affecting embryonic ovary development can be examined *in vitro* using an embryonic organ culture. This allows for the examination of the mechanisms underlying prophase I of meiosis up-to dictyate, germ cell nest breakdown, and follicle formation. In the mouse, the gonadal ridge can first be identified in E10.5 mouse embryos as a narrow strip of tissue on the urogenital ridges. From around E12.5 onwards, gonads can be sexed simply by

inspection, but if collected prior to this stage the embryo tissue must be collected and sexed by PCR. Various different embryonic culture systems have been set up over the past two decades with the aim of investigating the mechanisms of early ovary development. McLaren and Buehr (1990) developed one of the earliest embryonic ovary culture techniques, using intact organ culture system or ovary fragments from E10.5-12.5 gonadal ridges (McLaren and Buehr, 1990). Histological analysis demonstrated the appearance of some growing oocytes, classified as dictyate by the authors on the basis of histological examination. However the authors state that follicles did form but photomicrographs provided shown in the paper do not show this. Furthermore, the ovarian capsule was not well maintained, with germ cells protruding into the surrounding medium. This technique was then modified and used to examine the mesonephric contribution to testis development (Buehr et al., 1993), to examine the regression of the Mullerian duct (Donahoe et al., 1977), and again to investigate the mechanisms of sex determination in the indifferent gonad (Adams and McLaren, 2002). Dong *et al.* (2009) used a similar system and cultured ovaries from E12.5 which differentiated into germinal vesicle-stage oocytes, although they were unable to complete meiosis I (Dong et al., 2009).

There has been relatively little success in culturing pre-meiotic ovaries through both meiotic divisions to produce mature oocytes, other than through the use of invasive techniques such as nuclear transfer (Obata et al., 2002). There is, however, one recent study which has shown that pre-meiotic germ cells from mice can be cultured right through to the metaphase MII stage, undergo fertilisation and form 2-cell embryos. This was achieved by firstly culturing pieces of E12.5 mouse ovaries for 28 days in Activin-A supplemented medium, followed by isolation of oocytes within the tissue explants which were co-cultured with preantral granulosa cell monolayers for a further 6-7 days, and finally a maturation assay for 16-18h at the end of culture (Zhang et al., 2012).

Establishing a culture system whereby mouse ovaries can be cultured from a pre-meiotic stage to a mature, fertilizable oocyte and yield live pups, has proved challenging. A few studies have been somewhat successful, although the majority

involved either transplantation of the cultured ovary to the renal capsule of mice (Shen et al., 2006, Motohashi et al., 2009) or required a nuclear transfer into enucleated, fully grown oocytes from adult mice (Obata et al., 2002).

4.1.2. Embryonic ovary culture in reproductive toxicology

Out of the already relatively small number of embryonic and fetal ovary cultures that have been established, very few of these have been used to investigate potential reproductive toxicants on the developing ovary. The ones that have been carried out examined the effects of pre-natal mouse ovary exposure to 7,12-Dimethylbenz(a)anthracene (DMBA), a polycyclic aromatic hydrocarbon (PAH) found in cigarette smoke (Matikainen et al., 2002) and Bisphenol A (BPA) (Brieno-Enriquez et al., 2012), uranium (Angenard et al., 2011b), or human fetal ovary exposure to tamoxifen (Yu et al., 2014). In contrast, there are a vast number of studies that have investigated potential reproductive toxicants on the post-natal ovary *in vitro,* looking at effects of compounds such as cyclophosphamide, doxorubicin (DXR), BPA and Diethylstylbestrol (DES) (Iguchi et al., 1990, Xu et al., 2002, Devine et al., 2004, Miller et al., 2005, Desmeules and Devine, 2006, Myllymaki et al., 2005, Chen et al., 2007, Craig et al., 2010, Soleimani et al., 2011). It is therefore clear that post-natal ovary and follicle cultures have already made a useful and powerful contribution to the field of reproductive toxicology testing. However, the pre-natal phase of development includes the period of meiotic entry and early prophase I, which is a particularly vulnerable time in oocyte development. This has been repeatedly demonstrated during spermatogenesis in males, where different reproductive toxicants often result in chromosomal aberrations during prophase I of meiosis (Kuriyama et al., 2005, Marchetti et al., 2006, Geoffroy-Siraudin et al., 2010, Geoffroy-Siraudin et al., 2012, Liu et al., 2013). Furthermore, in females, the period of germ cell nest breakdown and formation of the primordial follicle pool is vulnerable to perturbations, which could have dire consequences for future fertility. It is therefore surprising that more efforts have not already been made to develop embryonic ovary culture systems to be used as an efficient and quick way to screen potential reproductive toxicants on the developing ovary. The lack of alternative test systems to supplement *in vivo* studies has frequently been commented on (Davila et

al., 1998, Jackson, 1998, Cortvrindt and Smitz, 2002). This has led to an increased demand for an adequate *in vitro* pre-natal ovary culture model that could be used to gain insight into the mechanisms of chemical exposure and to pinpoint potentially hazardous products on reproductive function, in a highly controlled manner.

4.1.3 The role of the synaptonemal complex in prophase I of meiosis.

As outlined in Section 1.1.1.2., meiosis is the specialised cell division that reduces the chromosome content of cells, to generate a haploid gamete containing one copy of each chromosome. Prophase I of meiosis, up to the point at which the oocytes arrest at dictyate, occurs during mammalian embryonic development. Homologous chromosomes start to pair up during the leptotene stage of prophase I, by finding their homologues and aligning their axes. The chromosomes require a proteinaceous structure, the synaptonemal complex (SC), in order to synapse. The SC becomes incorporated between the homologous chromosomes between leptotene and zygotene, and brings their axes together (Zickler, 2006). The SC is composed of three elements, the axial (SYCP2 and SYCP3), transversal (SYCP1) and central elements (SYCE1, SYCE2 and TEX12) (Fig. 4.1) (Baillet and Mandon-Pepin, 2012). Recombinase proteins DMC1 and RAD51 interact with the axial elements and assist in the recognition between the homologous chromosomes, facilitating their synapsis (Tarsounas et al., 1999). The SC forms by incorporating the transverse element into the SC as it forms a connection between the axial elements, thereby 'zipping' up the homologous chromosomes (Ollinger et al., 2010). By pachytene, the SC is fully formed along the chromosomes, resulting in full synapsis. The SC then begins to break down, and the germ cells arrest at the diplotene stage of prophase I.

SC assembly is commonly used to investigate chromosome behaviour and monitor the progression of prophase I in early oocytes. In the mouse embryo, SYCP3 expression is absent at E12.5, but appears in over 80% of female germ cells by E13.5. Mouse germ cells are therefore in pre-meiotic stage at E12.5 and enter prophase I of meiosis at E13.5 (Shen et al., 2006).

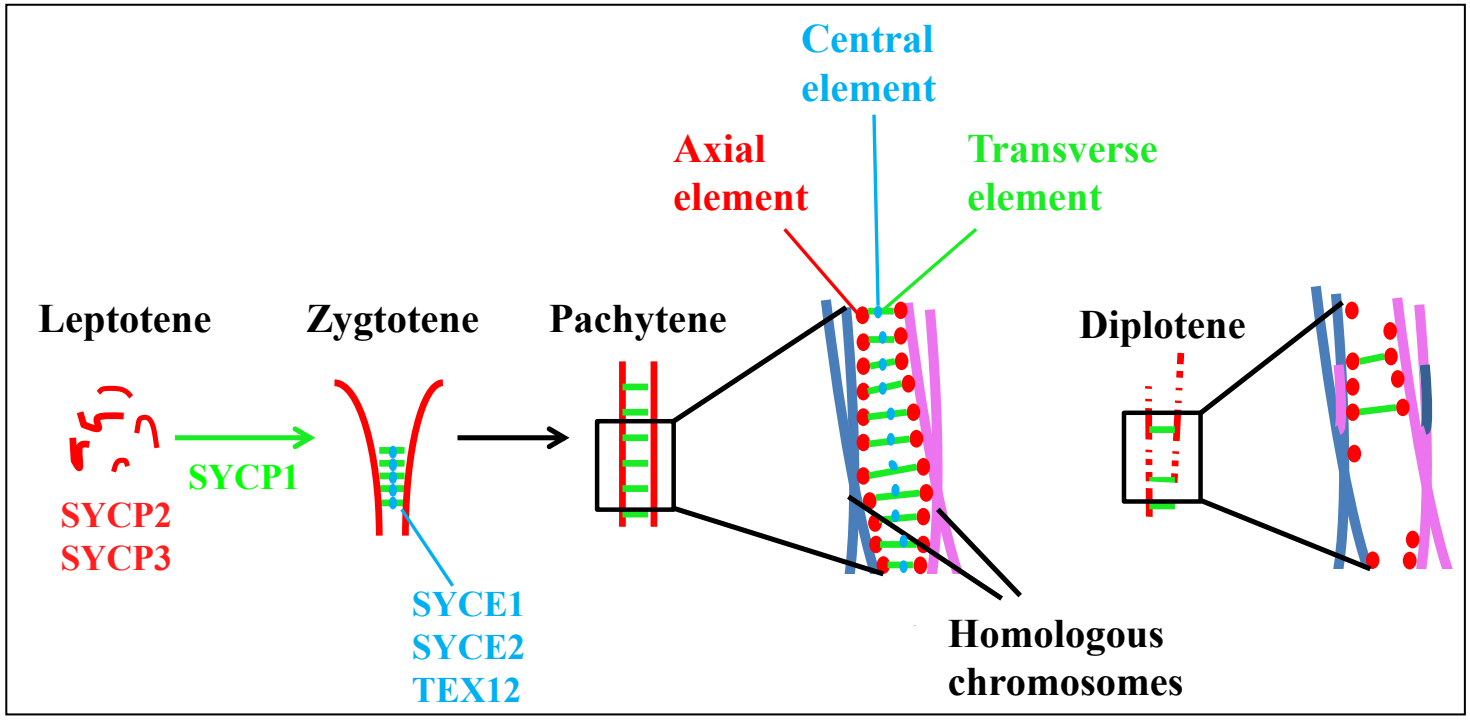


Figure 4.1. Graphic representation of SC formation and breakdown during prophase I of meiosis. The SC is composed of three elements, the axial, transversal and central elements. The axial element consists of SYCP2 and SYCP3 proteins, the transverse one consists of SYCP1 protein, and the central element of SYCE1, SYCE2 and TEX12.

4.1.4. Role of mesonephros in early ovary development

The indifferent mouse gonad develops along the inner surface of a rudimentary nephric organ, the mesonephros, during early embryonic development. In the male, the mesonephros eventually develops into the testicular efferent ducts whereas in the female embryo, it undergoes complete atrophy and regresses. During embryonic development, as the gonads differentiate into testes in males, the mesonephric duct becomes the Wolffian duct. The Wolffian duct contributes to the rete testes in the male, as well as the rete ovarii in the female. It has been suggested that cells from the mesonephros migrate to the gonad and contribute to the somatic environment during gonadal differentiation (Upadhyay et al., 1979, Wartenberg, 1981, Upadhyay et al., 1981). During testes development, interstitial cells derived from the mesonephros have been shown to be crucial for normal testis cord differentiation (Buehr et al., 1993). In the female, the mesonephros has also been suggested to be the source of somatic cells that invade the ovary and contribute to the ovarian interstitial and pre-granulosa cell populations, until the mesonephros eventually becomes invaded by a septum of connective tissue, separating it from the ovary (Upadhyay et al., 1979). Furthermore, the mesonephros has been shown to play a role in meiotic initiation of the mouse ovary by producing RA, a meiosis-inducing factor which diffuses to the ovary and induces the germ cells to enter meiosis (Bowles and Koopman, 2007b) (Section 1.1.1.2).

4.1.5 Growth factors involved in early ovary development

During embryonic development, the gonadal ridges release various soluble growth factors that influence PGC proliferation and survival (Godin et al., 1990). Leukemia inhibitory factor (LIF) and SCF (also known as KL) are commonly used factors in tissue culture media and stem cell media. LIF is a glycoprotein used primarily in stem cell cultures to suppress spontaneous differentiation of the cells, thereby maintaining stem cells in a long-term pluripotent state. It does, however, also have anti-apoptotic effects on PGCs in ovary culture (Pesce et al., 1993). SCF, amongst other factors, is implicated during embryonic development in promoting PGC survival and proliferation, as well as PGC migration toward the developing ovary (de

Felici, 2000). Furthermore, it promotes the initiation of primordial follicle growth (Durlinger et al., 2002) and plays a crucial role in primordial germ cell survival.

4.2 Aims

The aim of this work was to bridge, adapt and improve existing culture methods to generate a culture system that would span meiotic entry to meiotic arrest, germ cell nest break-down, follicle formation and initiation of follicle growth.

4.3 Materials and Methods

4.3.1 Method development of the embryonic mouse ovary culture

The initial culture was learned and adapted from a culture technique previously established by Dr. Ian Adams & Dr. Anne McLaren (Adams and McLaren, 2002). This culture system had been used to examine sex determination of the bipotential gonad, but oogenesis or follicle formation within the cultured ovaries had not been investigated.

4.3.2 Animals

CD-1 mouse breeding harems were set up in a cage (2 females with each proven male) and maintained on a 12:12h light/dark cycle. Females were checked the following morning from the presence of a copulation plug, which was designated as 0.5 dpc. E12.5 or E13.5 mice were obtained from pregnant CD-1 females and ovaries dissected as described below (Section 4.3.5).

4.3.3 Preparation of culture plate and agar block

2% agar (Sigma, A1296) was prepared in 1xPBS in a duran bottle and autoclaved before use. In a laminar flow hood the agar was poured into 10 cm petri dishes and allowed to set in the flow hood. Once set, the moulded agar was cut into equal 1cm x 1cm square blocks using a sterilised razor blade. The remaining agar was discarded. Each individual block was then transferred into a separate 33mm petri dish.

4.3.4 Preparation of the rich PGC medium (Rich medium)

The medium used was Dulbecco's Minimal Essential Medium (DMEM) (Life technologies, 41965039), supplemented with 10% Fetal Calf Serum (Thermo Fisher, SV30143/SV3060), 1% 200 mM L-glutamine (Invitrogen, 25030024), 0.2% 50 mM β-mercaptoethanol (Life technologies, 31350), 1% sodium pyruvate (Sigma, S8636), 1% 100 x penicillin/streptomycin (Invitrogen, 15140-122) and 1% of amphotericin B (Sigma, A9528) diluted to a stock solution of 2.5 mg/ml into DMSO (Sigma, D2650). This medium will from this point onwards be referred to as the rich medium. The medium was aliquotted into a 33mm petri dish containing an agar cube (See Section 4.3.3), ensuring the agar cube was well covered by medium, and was first left to incubate in the culture dish for 30 minutes at 37°C, allowing the medium to penetrate the block fully. The medium was then removed, replaced with fresh medium and left to equilibrate for another 30 minutes before ovaries were placed on the block.

4.3.5 Isolation of ovaries from mouse embryos

Pregnant females were collected and culled by cervical dislocation. The uterus was removed from the mother and placed in a petri dish containing 1xPBS at 4°C. An incision was made along the uterus to release the embryos, which were then dissected out of their gestational sacs and decapitated. Embryos were placed in a new petri dish containing fresh 1xPBS at 4°C and kept on ice. The remainder of the dissection was carried out under a microscope at 20-30x magnification. Since the stage of gestation was critical, embryos were staged by examining the shape of the hind limb bud (Fig. 4.2). A small ventral incision was made in the embryo, and the lungs, heart, bowel and liver were removed out of the peritoneal cavity. This left clearly visible the mesonephros (Mullerian and Wollfian ducts) and the attached genital ridges. At this embryonic stage, it is possible to distinguish the females from the males by visual examination, as the testis can be identified by a characteristic circuitous capsular vessel that will become the testicular artery, and by its transverse sex cords that will become the seminiferous tubules. The ovary on the other hand, is longer and thinner and lacks any of the vessels or cords visible in the male gonad. Female gonads, including the mesonephros, were dissected from each side and

placed in a fresh dish of 1xPBS. Unless stated otherwise, the mesonephros was removed using sterilised syringe needles. The ovaries were picked up using a 200 µl pipette and placed in the culture medium to be rinsed, ensuring that minimal PBS was transferred along with the ovary. The ovary was then placed on top of the agar block and the level of media was adjusted so that the media was at the same level as the top of the agar block. Too much media caused the ovary to float off the block, whereas too little resulted in the ovary drying out. Therefore the ovary needed to be cultured at the ideal level of media to ensure optimal media/air interface. All dissections were carried out in aseptic conditions under a laminar flow.

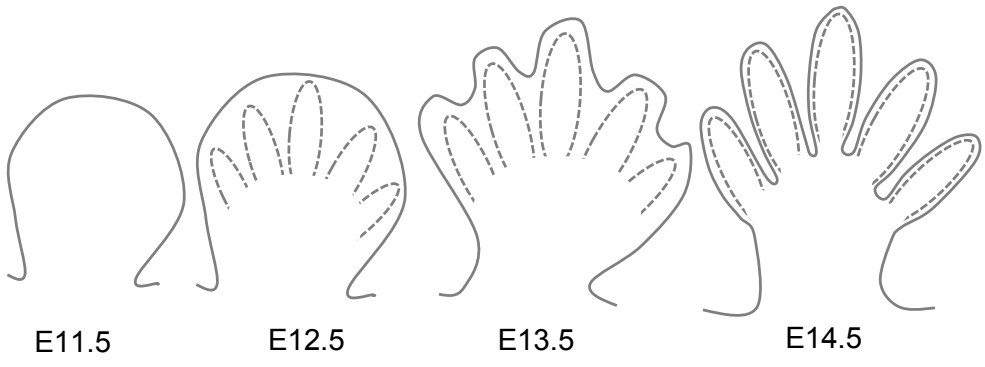


Figure 4.2. Limb bud stages used to measure gestational age of mouse embryos.

4.3.6. Method development

In order to improve and optimise the basic culture system described above, various different refinements were tried in order to support the germ cells in the embryonic ovaries from a pre-meiotic stage up to follicle formation and initiation of follicle growth. The different steps taken to optimise the culture system are outlined below.

4.3.6.1. Starting material for culture

Initial experiments included ovaries from both E12.5 and E13.5 mouse embryos, to determine the optimal starting point for the embryonic ovary culture. E12.5 ovaries were cultured for 6 or 12 days and E13.5 ovaries for 5, 6 or 11 days. It was hypothesised that 5-6 days in culture would bring the ovary to around the equivalent

time of birth *in vivo,* or around E18.5-E19.5. The longer 11-12 day cultures were also carried out, in order to investigate if the culture system would support follicle growth. 200 µl of fresh media was added to the culture every other day, and the media was completely replaced with fresh media after 6 days of culture. Embryos younger than E12.5 were not used since the gonads cannot be sexed by visual examination alone at this point. On the other hand, E14.5 and older embryos were not selected due to meiotic entry of oocytes at E13.5, since the culture starting point should, ideally, begin prior to meiotic entry of oogonia.

4.3.6.2. Hanging droplet and membrane culture

A hanging droplet culture was also attempted alongside the initial agar cultures, to investigate if the hanging droplet culture might show signs of being a more promising technique to culture embryonic gonads in small volumes. To set up a hanging droplet culture, a 96 well plate was used and 25 µl of rich medium (Section 4.3.4) was added to the lid above one of the wells. Dissected E12.5 ovaries were washed in 1xPBS and transferred into the droplets of media using sterilised dissection forceps to minimise transfer of PBS into the droplet. ddH_2O was added to any wells that did not contain an ovary, to avoid the droplets drying out. The lid was then carefully rotated and placed on top of the culture plate so that the ovaries and droplets hung from the lid above the culture well. E12.5 ovaries were also cultured for 6 days on a floating membrane in a 24 well plate containing 1 ml of rich medium, with half the medium replaced every other day.

4.3.6.3. Bridging of embryonic ovary culture & neonatal ovary culture

The embryonic gonad culture medium contains various growth factors that are essential for early oogonial development, but not required for later follicle growth. In order to test whether this rich medium was detrimental to later stages, a new system was tried, bridging the embryonic ovary culture with the neonatal culture system that was already established in the Spears lab, and using two different media (Sections 2.1.2 & 4.3.4). E13.5 ovaries were initially cultured for 6 days in the rich embryonic gonadal culture medium, and were then transported using a 200 µl pipette onto a

membrane in a well containing the simple (neonatal) culture medium for a further 6 days.

In a later experiment, ovaries were kept on the agar block for the full 12 days but the rich medium was replaced with the simple medium at earlier time-points, as it was hypothesised that 6 days of exposure to the rich culture medium was potentially still too long, hindering associations between the oocyte and granulosa cells and inhibiting appropriate follicle formation and/or follicle growth. The methodology steps are outlined in Figure 4.3.

4.3.6.4. Importance of β-mercaptoethanol for embryonic ovary health in vitro

β-mercaptoethanol is a widely used reducing agent in cell culture medium as it prevents the levels of oxygen radicals from rising to a toxic level. In order to establish whether β-mercaptoethanol was required for the early stages of embryonic ovary culture, 12 day cultures were set up as described above, with and without 0.02% β-mercaptoethanol in the medium.

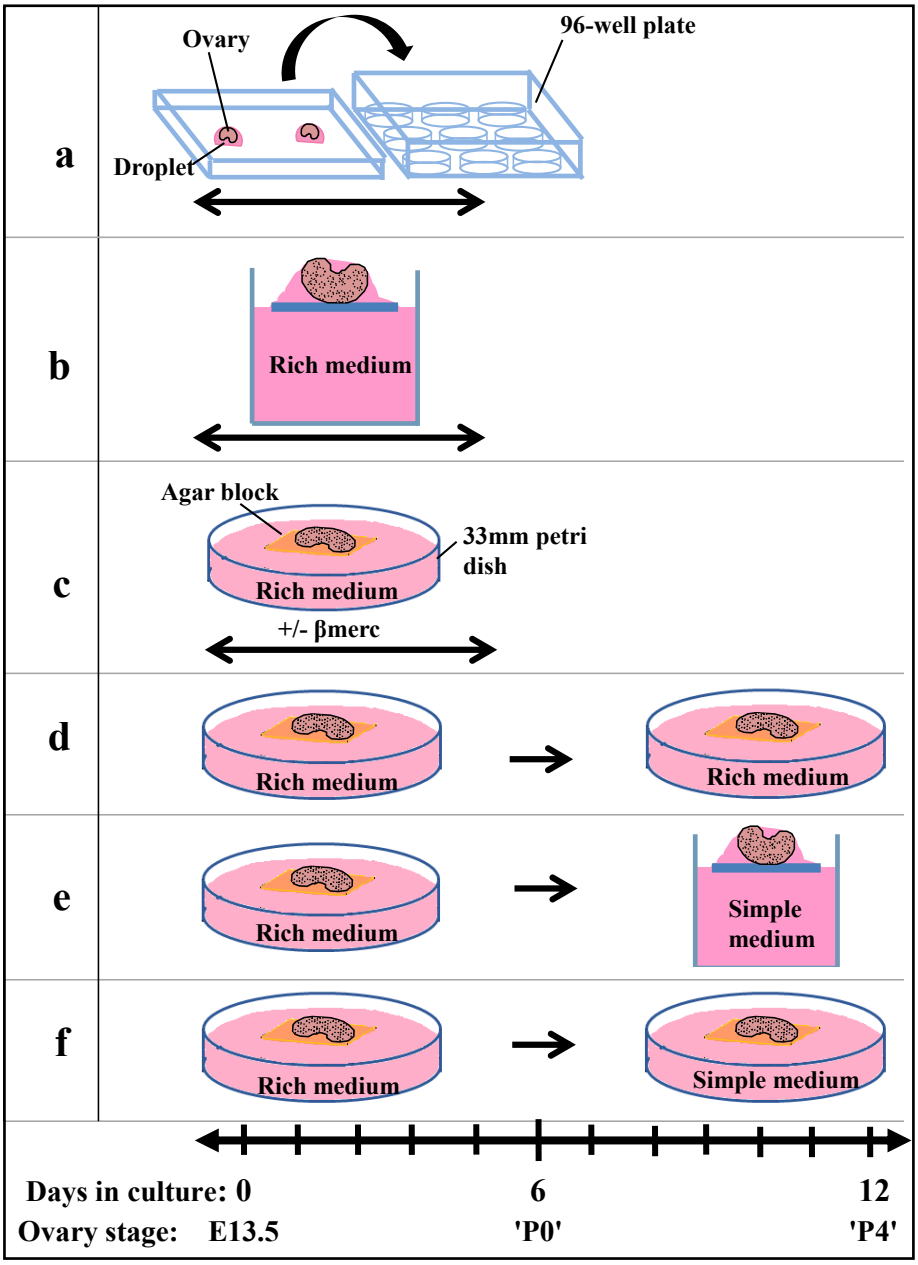


Figure 4.3. Bridging and developing the embryonic ovary culture system. Diagram outlining the different approaches taken to optimising the embryonic ovary culture system, using E12.5 and/or E13.5 ovaries. These include the hanging droplet culture (a), 6 day membrane culture with PGC media (b), 6 day agar culture with PGC media, with or without βmercaptoethanol (c), 12 day culture with PGC media (d), 12 day culture where ovaries are transferred from PGC medium over to the P0 membrane culture system after 6 days in culture (e) and a 12 day culture system where ovaries are left on the agar for the whole 12 days but the PGC media was changed for P0 media on days 3, 4, 5 and 6 (f).

4.3.6.5. Inclusion of mesonephros during culture

The mesonephros was originally dissected away from the embryonic ovary, as it regresses naturally around E16.5 *in vivo* (Upadhyay et al., 1979). However, due to its role in providing somatic cells to the developing ovary, later cultures were also set up where the mesonephros was not removed from the ovary prior to culture. The ovaries were cultured as outlined previously (Section 4.3.3-4.3.5) for 12 days on a 2% agar block where the rich medium was replaced by the simple medium at day 3 of culture. Cultured ovaries with the mesonephros removed and P4 un-cultured ovaries were used as controls.

4.3.6.6. Inclusion of serum second-half of culture period

Absence of serum from the culture medium of small pre-antral follicles has previously been reported to increase follicular apoptosis (Murray et al., 2001). Furthermore, unpublished observations from the Spears laboratory involving co-cultures between neonatal mouse ovaries and individual pre-antral follicles, (requiring 5% mouse serum in the culture medium) resulted in what appeared to be a slight increase in the number of in secondary follicles within the cultured neonatal ovaries (unpublished observation, Lisa Campbell, Federica Lopez & Norah Spears). Serum was therefore considered as a potentially good way to improve the culture system. Given that, the effect of adding serum to the culture medium was investigated to see if it could produce healthier primary follicles and potentially result in secondary follicle formation. E13.5 mouse ovaries were cultured for 3 days in the rich medium, 3 days in the simple medium, and finally a further 6 days in simple medium supplemented with 5% mouse serum (cultured on an agar block for the whole 12 days of culture).

4.3.6.7. LIF and SCF

SCF is frequently included in ovary culture due to its role in follicle initiation and maintenance of primordial follicle health (Farini et al., 2007). LIF is another commonly used factor in stem cell media for maintaining cells in a pluripotent state but is also an important factor for primordial germ cell survival and proliferation (Pesce et al., 1993). However, due to the inclusion of FBS in the medium which

already contains a variety of growth factors, these factors were not added to the embryonic ovary culture at this stage.

4.3.6.8. In vivo tissue collected for controls.

Ovaries from embryonic mice at E17.5, E18.5 and pups at P0 and P4 were collected to be examined alongside the cultured ovaries for an *in vivo* comparison in order to give a rough idea of the developmental stage of cultured ovaries.

4.3.6.9 Histology

Ovaries were fixed, processed, sectioned and H&E stained as outlined previously (See Section 2.2). Ovary and follicle morphology and health was then analysed histologically (See Section 2.3).

4.3.7 Histological analysis and follicle counts of E13.5 cultured ovaries (+/- mesonephros) compared with P4 *in vivo* ovaries

E13.5 ovaries were cultured with and without the mesonephros on an agar block for 12 days (3 days complex medium, 9 days simple medium). *In vivo* ovaries from P4 pups were collected for controls. Ovaries were fixed in formalin, washed in 70% ethanol, embedded in agar, processed, embedded, cut and H&E stained as described in Section 2.2. Every 6^{th} section from each ovary was photographed at x20 magnification. Follicles were counted blind and categorised as outlined in section 2.3.

4.3.8 Ability of cultured germ cells to progress through prophase I of meiosis.

Oocyte spreads were carried out on cultured E13.5 ovaries in order to investigate whether the germ cells within these cultured ovaries were capable of progressing through prophase I up-to the pachytene stage of meiosis I.

4.3.8.1. Sucrose solution

8.56g of sucrose were added to 50 ml of dH_2O. It was then aliquoted and frozen at -20˚C until use.

4.3.8.2. Hypotonic Extraction Buffer

The hypotonic extraction buffer was made by adding 3 ml 1 mM Tris, 10 ml 500 mM sucrose, 1 ml 0.5M EDTA and 0.5g sodium citrate to 100 ml dH_2O. The pH was then adjusted to 8.2, 10 ml aliquots were made and stored at -20˚C until use. Immediately before use, 5 µl 1M DDT and 50 µl PMSF were added to the 10 ml aliquot.

4.3.8.3. Paraformaldehyde fixative

A stock of 10% PFA was made by dissolving it in dH_2O with a couple of drops of 5M NaOH at 60˚C in a water bath. 1.5 ml 10 % Triton-X (Sigma, TT9284) was added for every 100 ml of PFA fix and pH was set to 9.2 using drops of 10mM Boric Acid.

4.3.8.4. Oocyte spread procedure

E13.5 ovaries were collected and cultured for 6 days on an agar block as outlined previously (Sections 4.3.3-4.3.5). Meanwhile, glass slides were boiled and left soaking in 1% PFA in a coplin jar before use. Following culture, ovaries were moved to M2 medium (Sigma, M7167) at 4˚C and kept on ice for up-to 30 minutes, while being transported. They were washed in 1xPBS at 4˚C and transferred into a hypotonic extraction buffer for 15-30 minutes. 10 µl of the 500 mM sucrose solution was placed on a glass slide along with one ovary from the PBS wash, and the ovary was pierced with a needle for approximately 5 minutes. Around 10 µl of this solution (now also containing a suspension of cells from the ovary) was picked up and dropped in the top corner of a slide that had been removed from the fixative, with its edges having been wiped with a tissue. The drop was then 'zig-zagged' down the fixative-coated slide so that the suspended cells spread out over the slide. The slide was placed in a humid chamber with hot tap water and left over-night at room temperature. The next morning, the lid on the humid chamber was opened and slides allowed to air dry for 1-2 hours. Once dry, slides were placed into a coplin jar containing 1XPBS at 4 ˚C for immunostaining.

4.3.8.5. Sycp1 and Sycp3 immunostaining

A blocking solution was made by adding 100 µl of 1.5%BSA (0.15g BSA in 10 ml 1xPBS), 10 µl 10% Tween and 50 µl goat serum into 840 µl 1xPBS. Slides were

washed in 1xPBS for 5 minutes and placed in a humid chamber. 50 µl of the block was added to each slide, a plastic cover-slip was placed on top and left for 1 hour at room temperature. The plastic cover-slip was then removed, the block was drained off and the primary antibodies were diluted in the block. The primary antibodies used were Sycp1 (ab15090-100, Abcam) and Sycp3 (sc-74569, Santa Cruz), both diluted 1:200 in the blocking solution. 50 µl per slide of the primary antibodies were added to the slide, a new plastic cover-slip was placed on top and the slides were incubated in the humid chamber for a further 2 hours at room temperature. Following incubation, the slides were washed 3x5 minutes in 1xPBS. The secondary antibodies, Anti-Rabbit Fluor 488 (A-11008, Invitrogen) and Anti-Mouse Alexa Fluor 594 (A-11005, Invitrogen) as well as DAPI were all diluted in block (1:500), adding a cover-slip and incubated in the dark at room temperature for 1 hour. Slides were washed for 3x5 minutes in PBS before 2 drops of PD mountant was added and left at room temperature for 10-30 minutes, before Vectashield soft-mount was added. A glass cover was carefully placed on top and once dry, the slides were then sealed with nail varnish and stored at 4˚C until visualised.

4.3.8.6. Visualision of immunofluorescence.

The immunofluorescence was visualised with an imaging system comprising of a Hamamatsu Orca AG CCD camera (Hamamatsu Photonics (UK) Ltd), Zeiss Axioplan II fluorescence microscope with Plan-Neofluor or Plan apochromat objectives, a lumen 200W metal halide light source (Prior Scientific Instruments, Cambridge, UK) and Chroma #89014ET single excitation and emission filters or Chroma #89000ET single excitation and emission filters (Chroma Technology Corp., Rockingham, VT). A piezoelectrically driven objective mount (PIFOC model P-721, Physik Instrumente GmbH & Co, Karlsruhe) was used to control the movement in the z dimension. Volocity (Perkinelmer Inc, Waltham, MA) was used to perform hardware control as well as to capturing and analyse images. The images were deconvolved using a calculated PSF (point spread function) with the constrained iterative algorithm of Volocity (Perkinelmer Inc, Waltham, MA) and a single 2D image was generated using Fiji imaging software (Cardona, 2012).

4.3.8.7. Analysis of pachytene nuclei frequency

Slides were carefully scanned and any nuclei in leptotene, zygotene or pachytene were imaged. Nuclei in any of these three stages of prophase will appear differently to other non-meiotic cells (or cells in pachytene) since the immunofluorescence detects the SC, which is not present during mitosis and has partly broken down by the diplotene stage. The nuclei were then analysed and categorised according to their stage of meiosis (Fig. 4.4)

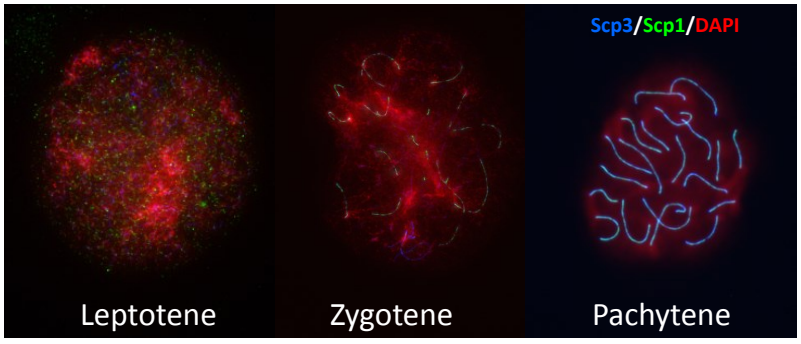


Figure 4.4. Representative nuclei for each stage of early prophase. At leptotene, chromosomes condense and very little synapsis is observed By zygotene, synapsis begins to be visible although it is incomplete. At pachytene, full synapsis between homologous chromosomes occurs.

4.3.9 Biotin tracer study

In order to visualise and analyse follicle architecture and basement membrane formation in the cultured embryonic ovaries, permeability of the ovaries was assessed by using a biotin tracer study. This study was adapted and modified from previous studies that had aimed to examine the integrity of the blood-testis barrier and the presence of functional TJs in the ovary, in which the authors had injected and/or cultured gonads with a biotin tracer (Mora et al., 2012, Elkin et al., 2010).

4.3.9.1 Ovary culture with biotin tracer

E13.5 and P0 ovaries were collected and cultured for 12 and 6 days respectively (for embryonic ovary culture, see Sections 4.3.3-4.3.5, for P0 ovary culture, see Section

2.1.) EZ-Link-Sulfo-NHS-LC-Biotin (Thermo Scientific, 21335) was diluted in 1xPBS (containing 1mM $CaCl_2$) at a concentration of 5 mg/ml. 500 µl of the biotin/PBS solution was added to 2 wells in a 24 well culture plate, a membrane placed on top and the PBS allowed to warm to 37˚C in an incubator before the ovaries were added (Section 2.1). At the end of the culture period, ovaries were transferred from the culture to the well containing the Biotin/PBS solution and placed back in the incubator for 30 minutes, to allow the biotin to perfuse. The ovaries were then moved off the membrane and placed in the well to allow them to be fully immersed in the solution for a further 5 minutes. The same 30 minute Biotin/PBS culture was carried out for ovaries from P4 pups, as a positive control.

4.3.9.2. Histology

The ovaries (cultured and P4 in vivo ovaries) were fixed in 4% buffered formalin for 24 hours, followed by a wash in 70% ethanol. They were then processed, embedded and sectioned as outlined in section 2.2.

4.3.9.3. Immunofluorescence

Fluorescence was used for the detection of biotin. Slides were dewaxed and rehydrated (See Section 2.4.1) followed by a one hour incubation with a NGS/BSA/PBS blocking solution as outlined in section 2.4.3. The primary antibody incubation step was omitted since the biotin was already within the ovaries following culture. To detect the biotin, slides were incubated at RT for 1 hour in the dark, with a solution containing Streptavidin 488 fluorescent antibody (Alexafluor, Invitrogen, UK #S11223). Streptavidin has a very high binding affinity for biotin. The Streptavidin antibody was diluted at 1:200 following the same steps as outlined in section 2.4.6. Slides were then washed in ddH_2O and PBS + Triton X (while kept in the dark) followed by a 20 minute incubation with DAPI counterstain (Invitrogen, D3571), diluted 1:10000 in ddH_2O. The slides were then mounted with Vectashield (Vector, H-1400) and a glass cover carefully placed on top. Once dry, the slides were sealed with nail varnish and stored at 4˚C until visualisation. Slides were later visualised and photographed using a Leica A6000 fluorescent microscope.

4.3.10 Antibody detection of laminin-α1

Immunohistochemistry was carried out to for Laminin, the major component of the basal lamina, on 13.5 ovaries that had been cultured for 12 days (Sections 4.3.3-4.3.5), cultured P0 ovaries (Section 2.1) and uncultured ovaries from P4 pups, to investigate whether the cultured ovaries support the appropriate establishment and localisation of the basal lamina. Laminin was detected in the ovary using DAB as an amplified step. Firstly, slides were blocked and the primary antibody (Laminin: Abcam, ab11575) was diluted 1:300 in the blocking solution, before being applied to the slides. The slides were incubated overnight at 4 ºC in a humidified chamber. Slides were washed and incubated for 1 hour with the secondary antibody solution, diluted 1:200 (goat anti-rabbit biotinylated antibody, Dako). Slides were then washed and incubated for 30 minutes with the ABC kit (Vectastain, PK6100) before being washed and incubated with DAB (Vektor, SK4100) until brown staining appeared (Section 2.4.6). Slides were then counter-stained, cover-slipped and imaged (Section 2.4.6).

4.3.11 Statistical analysis

Graphpad Prism was used for all statistical analysis of follicle number and distribution between cultured ovaries and *in vivo* ovaries. Data normality was assessed using Kolmogorov Smirnoff tests. Where data was not normally distributed, the Kruskal-Wallis non-parametric test was used to analyse the data. This was followed by a Dunns post-hoc test if the Kruskal-Wallis test showed significant difference. Where the data was normally distributed, a one-way ANOVA was used to determine if there were any significant differences between cultured ovaries and P4 ovaries, followed by the Bonferroni post-hoc test if the ANOVA showed a significant difference.

4.4 Results

4.4.1. Method development

4.4.1.1. Starting material for culture

Ovaries from E12.5 and E13.5 mouse embryos were dissected and used as starting material. These stages were selected due to the entry of oogonia into meiosis around E13.5. Ovaries were initially cultured for 5-6 days on 2% agar in the rich medium (Sections 4.3.3-4.3.5). Ovary sections were H&E stained and analysed. An E17.5 ovary was also dissected, fixed, processed, sectioned and stained in the same manner, to be compared with the cultured ovaries.

E13.5 ovaries that had been cultured for 6 days contained larger cells with oocyte-like appearance and with nuclei that appeared to be in the dictyate (Fig. 4.5c). These cells looked morphologically very similar to those observed in the E17.5 ovary, although much fewer of these germ cells were observed in the cultured ovary (Fig.4.5d). E12.5 ovaries cultured for 6 days, and E13.5 ovaries cultured for 5 days showed no signs of any larger oocyte-like cells, with the germ cells appearing to still be in the zygotene/pachytene stage of meiosis (Fig. 4.5a and 4.5b). E13.5 ovaries were therefore chosen as a starting point for future cultures. This also allowed the mesonephros to be dissected away with more ease than was the case with the E12.5 embryo, with a lower chance of tearing the ovary during mesonephros removal.

Early method development steps also involved attempts to culture E12.5 ovaries in a hanging-droplet culture or on a floating membrane in a 24 well plate containing the rich medium for 6 days (Fig. 4.6). Neither the hanging droplet culture nor the membrane culture resulted in oocyte-like cells forming, unlike those observed in E13.5 ovaries cultured on agar (Fig. 4.5c).

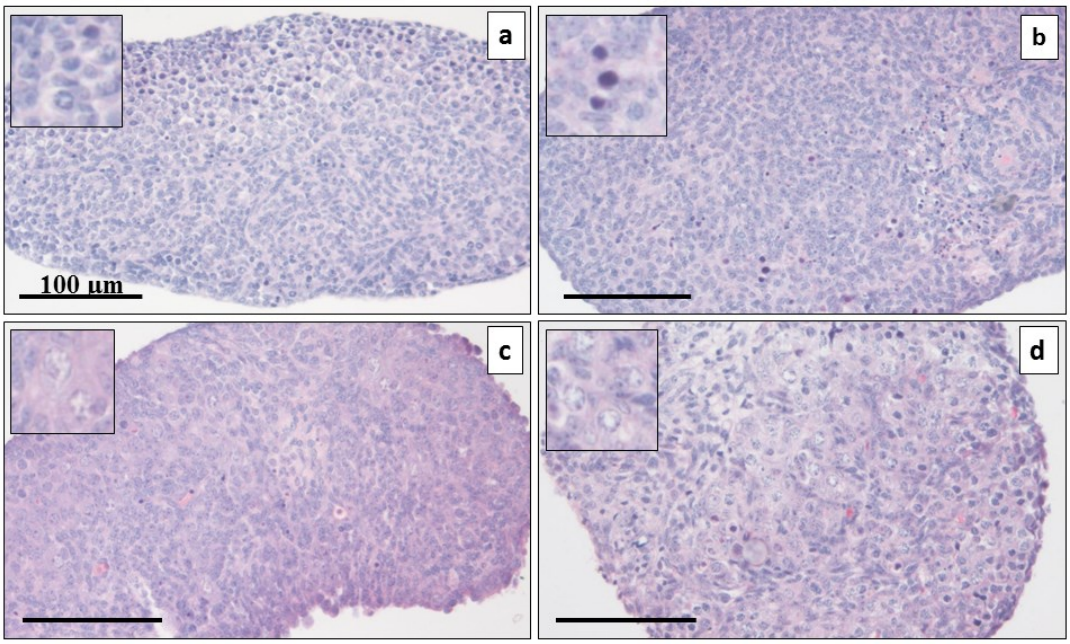


Figure 4.5. Histological analysis of embryonic ovaries cultured for 5-6 days. E12.5 ovaries were cultured for 6 days (a), E13.5 ovaries were cultured for 5 & 6 days (b, c). E17.5 in vivo ovary was used as a control (d). Oocyte-like cells with nuclei that appeared to be in the diplotene stage of meiosis were observed in E13.5 ovaries that had been cultured for 6 days in the embryonic ovary culture system (c). These oocytes had many similar characteristics to those observed *in vivo* at E17.5 (d).

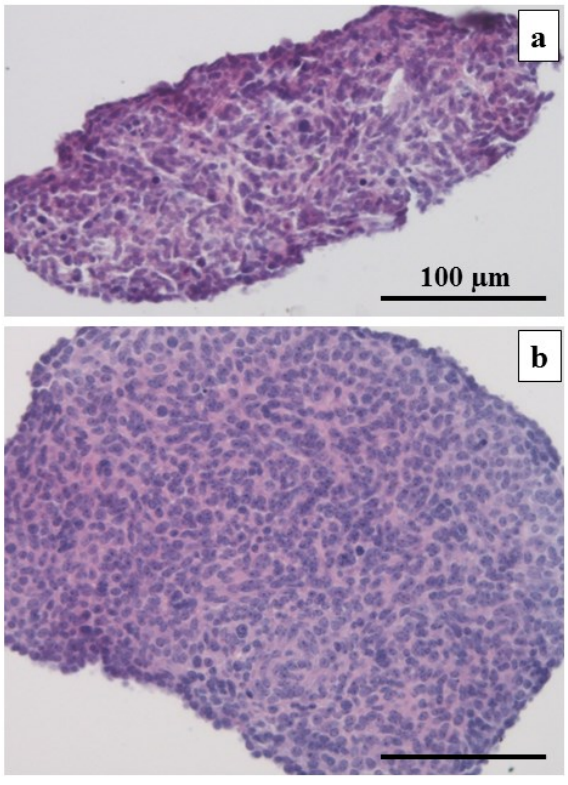


Figure 4.6. Representative photomicrographs of E12.5 ovaries cultured in an agar droplet and on a membrane. E12.5 ovaries were cultured for 6 days in a hanging droplet culture (a) or on a membrane floating on PGC media (b). Although the ovaries cultured on a membrane appeared healthier than the ones in the hanging droplet culture, neither ovary appeared to develop any oocyte-like cells.

4.4.1.2. Bridging embryonic and P0 ovary cultures

Following on from the shorter 5-6 day cultures, E13.5 (Fig. 4.7a) and E12.5 (Fig. 4.7b) ovaries were then cultured for 12 days on 2% agar blocks in petri dishes containing the rich medium, and examined histologically. Histological analysis of the ovarian structures that developed within these ovaries demonstrated that, although they were smaller, they resembled *in vivo* ovaries in terms of general morphology (Fig. 4.7). Following the 12 day culture, ovaries contained both small non-growing oocytes within primordial follicles as well as growing oocytes, but the majority of oocytes did not appear healthy, particularly in the E12.5 cultures (Fig. 4.7a-b). Several germ cell nests and bi-nuclear oocytes were observed in cultured E12.5 ovaries (see asterisk in Fig.4.7b). Many oocytes contained large vacuoles, with the nucleus becoming displaced to one side of oocyte. Furthermore, granulosa cells did not appear to form any visible associations with the oocytes, where they failed to form the appropriate coronal arrangement around the oocytes. This was further characterised by a lack of basement membrane formation around the follicles.

Neonatal mouse ovaries cultured in the presence of the simple medium are capable of forming healthy follicles which can initiate growth. In order to test the hypothesis that the various growth factors present in the richer medium were disruptive to follicle formation and growth, it was therefore attempted to bridge the embryonic ovary culture and the P0 culture system (Section 2.1.2). It was hypothesised that this would improve oocyte health and follicle formation. E13.5 ovaries were initially cultured for 6 days on an agar block with the rich medium, followed by a transfer to the neonatal ovary culture system, where they were cultured for a further 6 days on a membrane floating on the simple medium (Section 2.1.2). Although this culture system showed fairly large improvements in oocyte health, several atretic follicles were still observed in these ovaries (Fig 4.7c, white arrow). Furthermore, there were large areas of unevenly distributed and unhealthy stroma (See Figure 4.7b, circle) and the ovarian capsule was not well maintained, with the ovary edges appearing frayed and containing necrotic cells (See Figure 4.7c, black arrow). This, as well as the fact that the ovaries spread out into a thin layer on top of the membrane, suggests that the ovaries were unable to maintain their structural integrity on a tough surface,

such as the membranes, and perhaps required the more permissive and gentle support of the agar. Therefore, another culture was attempted where E13.5 ovaries were kept on the agar block for the whole 12 days of culture, where the rich medium was replaced with the simple medium after 6 days of culture, roughly around the point of which follicles would begin to form. At the end of culture, these ovaries appeared considerably healthier than the ones that had been cultured on membranes for the latter half of the culture duration (Fig. 4.7d). The oocytes and stroma appeared healthier, with the ovary capsule also remaining intact. However, the follicles still lacked the appropriate BM formation, and germ cell nests were also observed (Fig. 4.7d, circle and insert).

To investigate if the embryonic ovary might benefit from an even earlier removal of the rich medium containing growth factors, the rich medium was replaced with the simple medium at a series of earlier time-points. E13.5 ovaries were cultured as outlined before, for 12 days, but the rich medium was replaced with the simple medium at different stages of culture, on days 3, 4, 5 and 6 (Fig. 4.8). When the media was replaced at the later stages (days 4, 5 and 6), the basement membranes did not appear to establish appropriately (Fig. 4.8a-c). However, when the media was replaced on day 3 of culture, the BM within the ovaries became clearly visible and the granulosa cells exhibited a more physiological association with the oocytes, forming a clear layer of granulosa cells around the oocytes (Fig. 4.8d, black arrows).

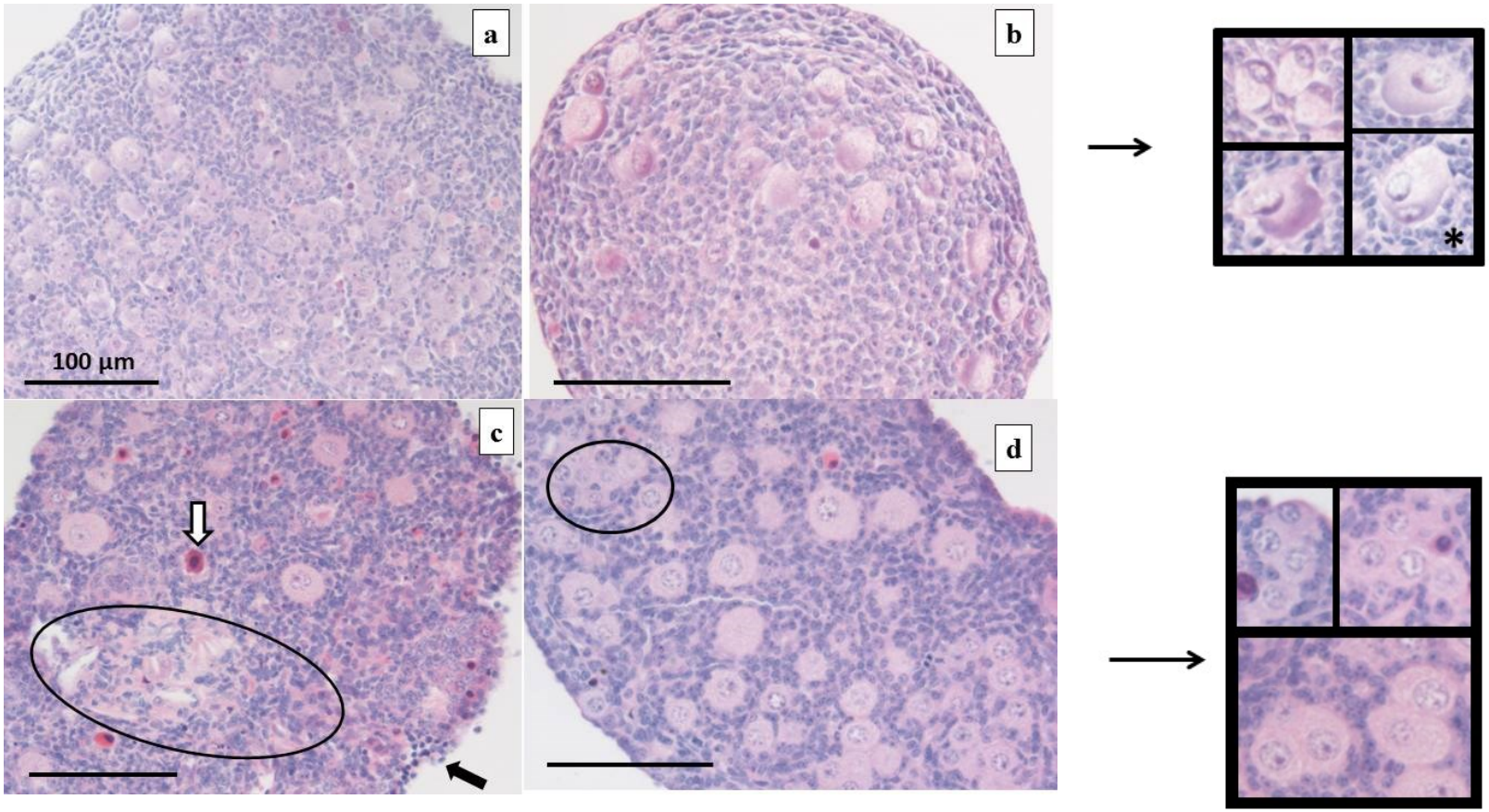


Figure 4.7. Photomicrographs of embryonic ovaries cultured for 11-12 days outlining different methodologies. E13.5 (a) and E12.5 ovaries (b) cultured for 12 days on agar with rich media, producing largely unhealthy oocytes (b, insert) and some MOFs (b, insert: asterisk). E13.5 ovaries cultured for 6 days with rich media on an agar block & transferred onto a membrane with simple media another 6 days (c). These ovaries looked better, but still contained many unhealthy oocytes (c, white arrow), areas of necrosis (c, circle) and with badly maintained ovarian capsule (c, black arrow). E13.5 ovaries kept on agar for the duration of the culture, with culture medium changed to simple medium on day 6 of culture produced the healthiest looking oocytes, although granulosa cell layers and basal lamina formation still failed to establish appropriately & germ cell nests appeared to be present (d, see circle & insert).

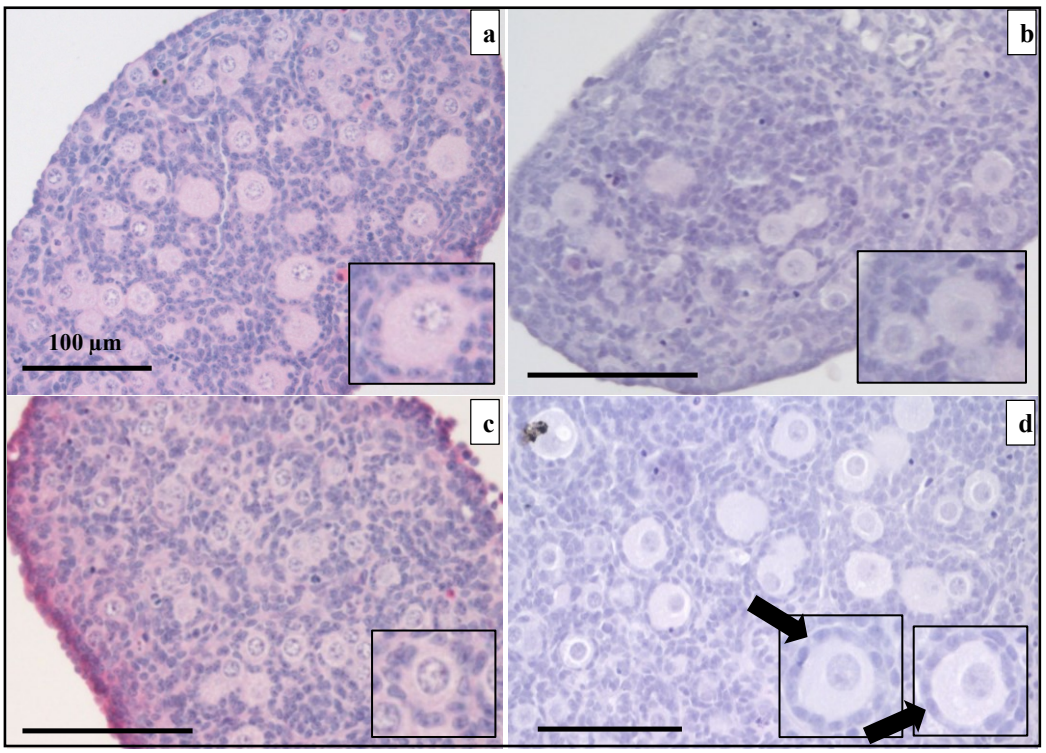


Figure 4.8. Photomicrographs of cultured ovaries changed over to P0 media at different time-points. E13.5 ovaries were cultured for 12 days with the rich medium initially, which was then replaced with the simple medium on different stages of culture: at day 6 (a), day 5 (b), day 4 (c) and day 3 (d). When the rich medium was replaced by the simple medium at day 3, basement membranes appeared better established than if replaced at later stages of development (d, black arrows).

4.4.1.3 Importance of β-mercaptoethanol for the embryonic ovary health

β-mercaptoethanol is a commonly used reducing agent in cell culture as its ability to scavenge hydroxyl radicals prevents the build-up of toxic levels of oxygen radicals. In order to investigate its importance in the embryonic ovary culture, E13.5 ovaries were cultured for 6 days with and without β-mercaptoethanol. Ovaries cultured without β-mercaptoethanol had fewer germ cells at the oocyte stage when compared with those cultured with β-mercaptoethanol (See Figure 4.12). As a result, β-mercaptoethanol was included in the PGC culture medium in all future experiments.

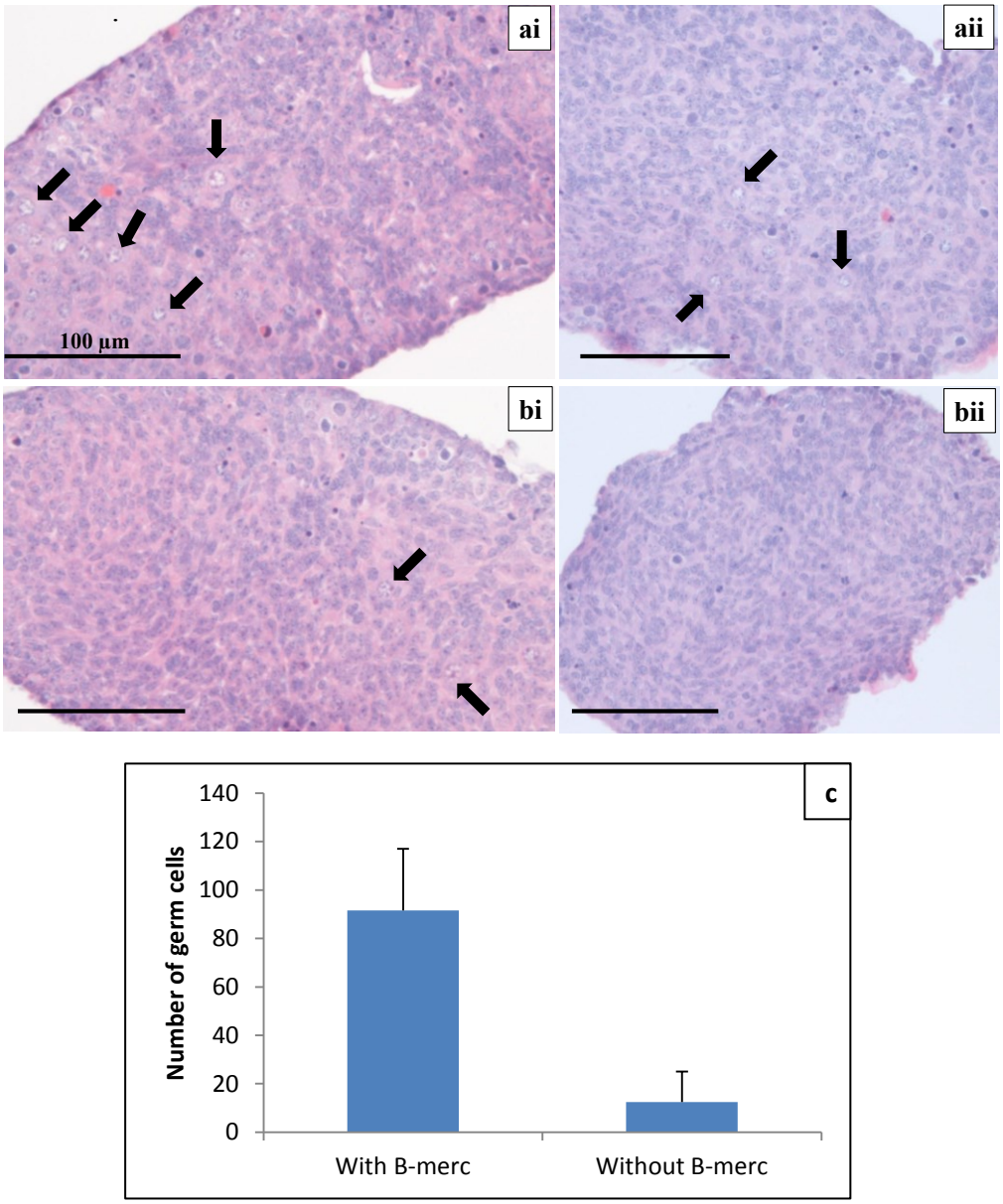


Figure 4.9. Photomicrographs of representative E13.5 ovaries cultured with (a) and without (b) β-mercaptoethanol, and a histogram outlining the number of oocyte-like cells counted in ovaries cultured with or without β-mercaptoethanol (c). At the end of the 6 day culture, ovaries that had been cultured with β-mercaptoethanol appeared to have relatively more larger oocyte-like cells (ai and aii: black arrows) than did ovaries cultured without it (bi: black arrows). Some of the ovaries deprived of β-mercaptoethanol in culture were found to be completely void of such cells (bii). When quantified, ovaries cultured without β-mercaptoethanol were found to have an average of 12.5 oocyte-like cells, compared with 91.5 when β-mercaptoethanol was kept in the medium. Bar denotes ±SEM. N= 2.

4.4.1.4 Effect of mouse serum during second half of the culture period

E13.5 mouse ovaries were cultured for 12 days as previously described (Sections 4.3-4.5) on 2% agar for 3 days in rich medium and 9 days in simple medium (n:3). Mouse serum was added during the second half of the culture period (or from the 6th-12th day of culture). No differences were observed between ovaries when examined histologically (Fig 4.10). Mouse serum was therefore omitted from future experiments.

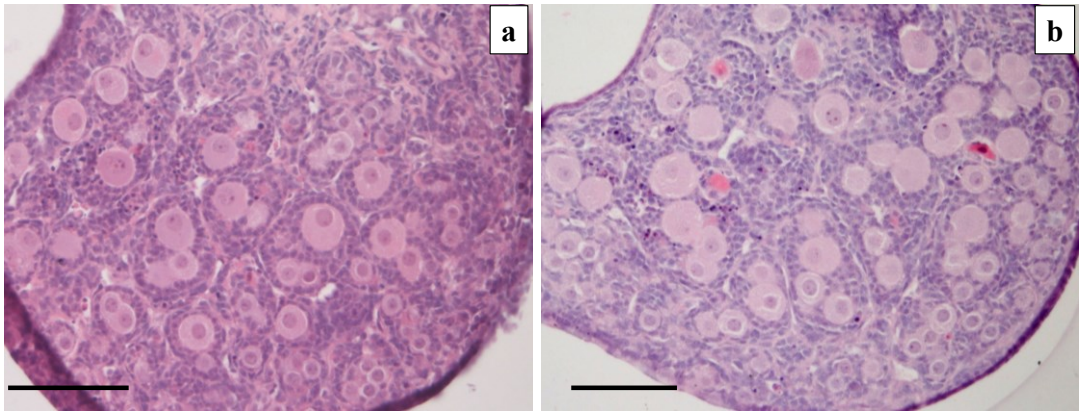


Figure 4.10. Photomicrographs of cultured E13.5 ovaries with or without mouse serum during second half of culture. E13.5 ovaries were cultured for 12 days (3 day PGC media, 9 day P0 media) either with no mouse serum (a) or with mouse serum during the latter 6 days of culture (b). No obvious differences were observed histologically between the ovaries.

4.4.1.5 Histological analysis of E13.5 cultured ovaries compared with P4 ovaries.

E13.5 ovaries that had been cultured for 12 days on agar (3 days rich medium, 9 days simple medium), with (n=5) and without (n=3) the mesonephros, as well as ovaries from P4 mouse pups, (n=3) were fixed, processed and cut (Section 2.2). Every 6th ovary section was H&E stained and counted (Section 2.3).

E13.5 ovaries, cultured for 12 days either with or without the mesonephros, demonstrated a typical follicular distribution pattern throughout the ovary with non-growing primordial follicles surrounding the periphery of the ovary, and growing

transitional and primary oocytes in the inner part of the cortex and medulla (Fig. 4.11). However, follicle numbers were significantly reduced however at the end of the 12 day culture when compared with the P4 ovary. The average number of follicles in the P4 ovaries was 1563±138, compared with 672±109 follicles in ovaries cultured with the mesonephros, and 536±304 in ovaries cultured without the mesonephros (Fig. 4.12a). Despite this reduction in follicle pool, there were still large numbers of follicles left in the cultured ovaries and the follicles appeared morphologically normal and healthy with only a slight, non-significant increase in unhealthy follicles in the cultured ovaries compared with P4 ovaries (3% increase without and 3.6% increase with mesonephros, p=0.38) (Figure 4.12b).

There was a significant reduction in the number of primordial and secondary follicles in cultured ovaries compared with *in vivo* (primordial follicle numbers: 535±114 (*in vitro* +meso), 359±290 (*in vitro –meso)* vs. 1389±120 *in vivo.* Secondary follicle numbers: 1.2±0.9 (*in vitro* +meso) and 2.1±5.1 (*in vitro* –meso) vs. 13±1 *in vivo).* Transitional and primary follicle numbers remained unaffected (Fig 4.13). However, when the follicle ratios were examined, by calculating the percentages of each follicle type present in each ovary, the cultured ovaries contained follicles at stages in comparable ratios to those in P4 in vivo ovaries (77.1% primordial, 17.0% transitional and 5.6% primary in cultured ovaries with mesonephros, 65% primordial, 25% transitional and 9% primary in cultured ovaries without the mesonphros vs. 88%, 7.8% and 2.8% respectively*, in vivo*) (Fig 4.14). Although there was no significant difference between ovaries cultured with and without the mesonephros, ovaries consistently showed trends towards the *in vivo* method when the mesonephros was left attached. Given that, ovaries cultured with the mesonephros were considered the better method for future cultures.

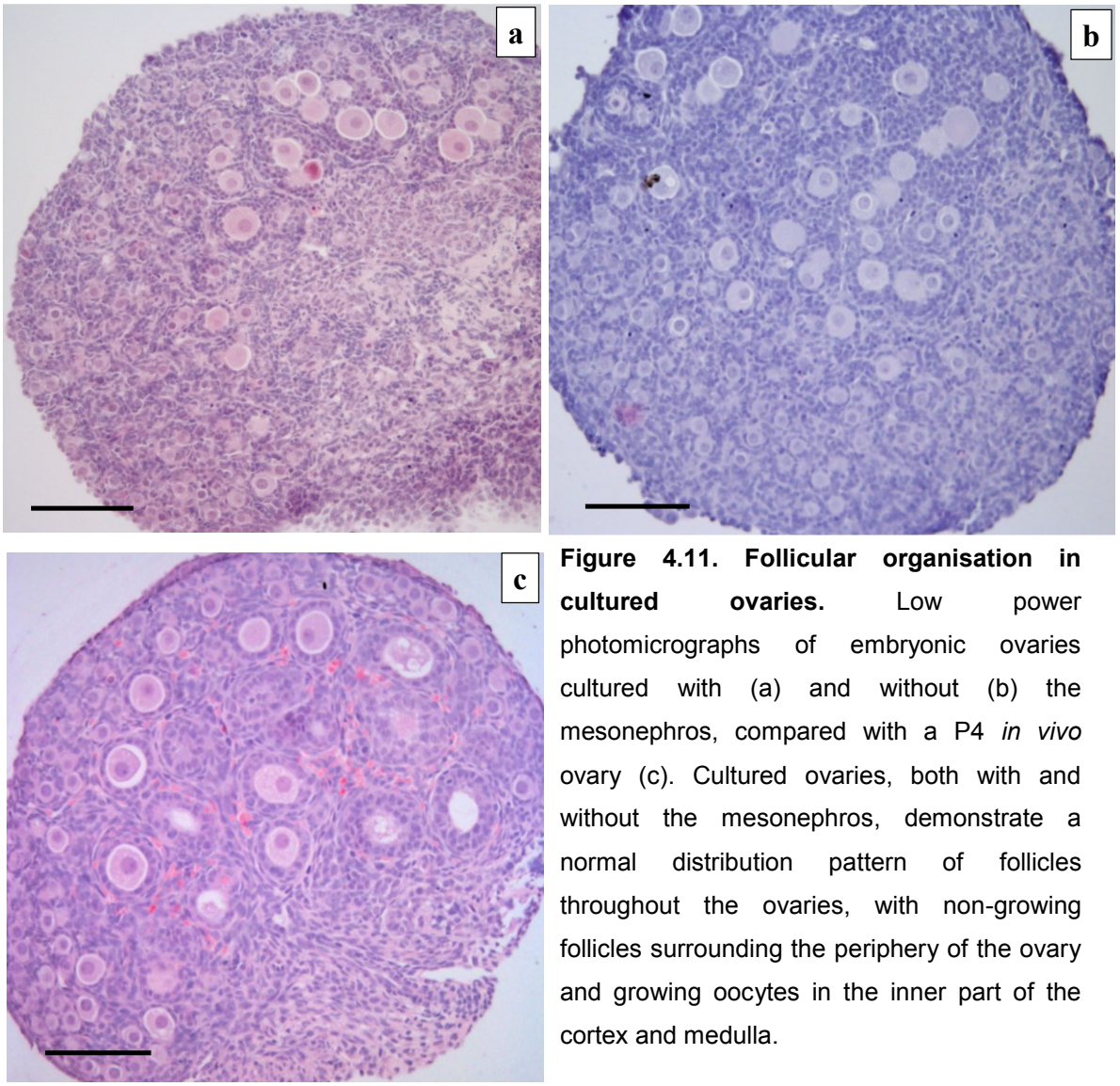


Figure 4.11. Follicular organisation in cultured ovaries. Low power photomicrographs of embryonic ovaries cultured with (a) and without (b) the mesonephros, compared with a P4 *in vivo* ovary (c). Cultured ovaries, both with and without the mesonephros, demonstrate a normal distribution pattern of follicles throughout the ovaries, with non-growing follicles surrounding the periphery of the ovary and growing oocytes in the inner part of the cortex and medulla.

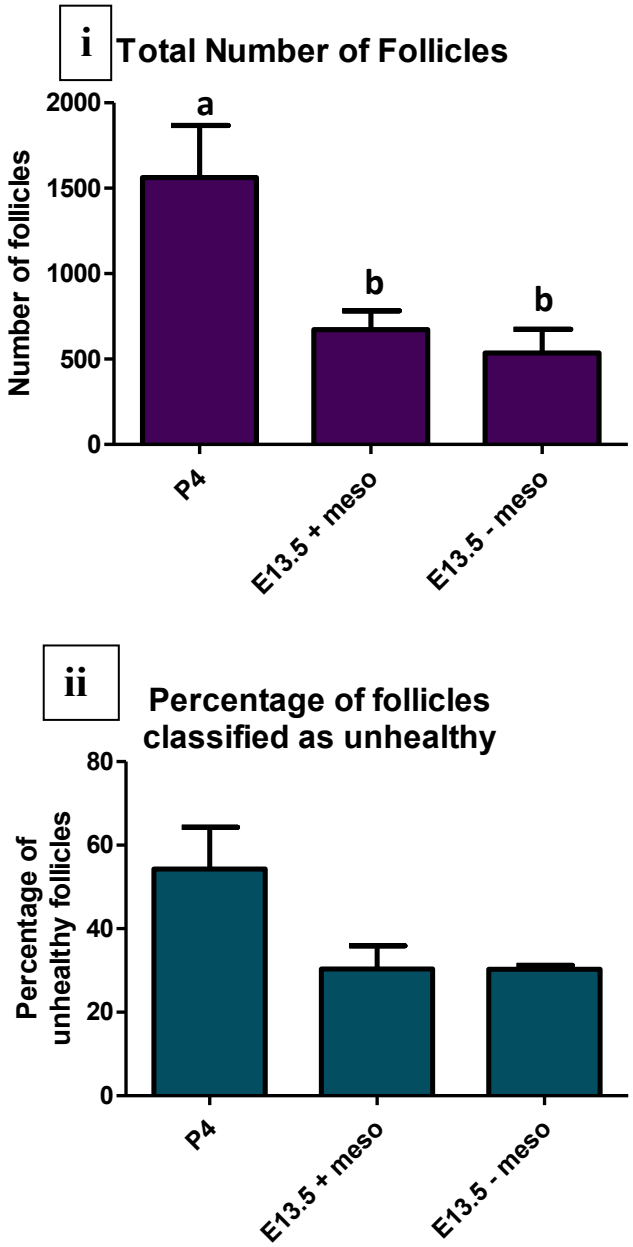


Figure 4.12. Total follicle numbers (i) and percentage of unhealthy follicles (ii) within cultured E13.5 ovaries, with and without mesonephros, compared with P4 un-cultured ovaries. E13.5 ovaries had significantly lower follicle numbers at the end of cultured than P4 ovaries ($p<0.05$) (i), but follicle health within these ovaries was not significantly affected (ii). Bars denote mean + sem; n=5 for +meso, n=3 for P4 and - meso. Means with different letters are significantly different ($p<0.05$).

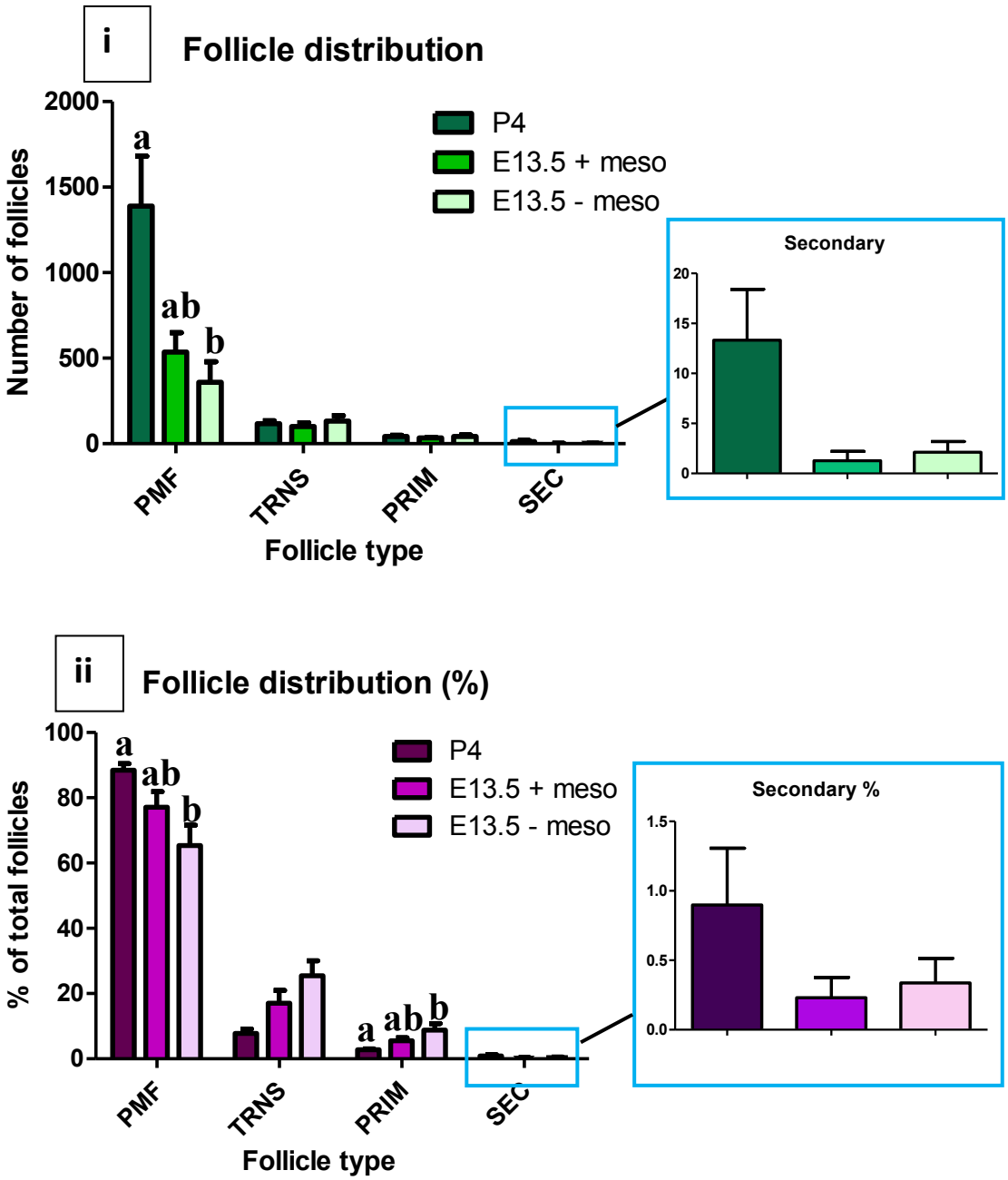


Figure 4.13. Distribution of follicle types in embryonic ovaries cultured with or without the mesonephros. Cultured E13.5 ovaries had significantly lower primordial ($p<0.05$) follicle numbers at the end of culture compared with P4 ovaries. Transitional, primary and secondary follicle numbers were not affected by the culture system (i). Cultured E13.5 ovaries had significantly lower percentage of primordial and secondary follicle at the end of cultured compared with P4 ovaries ($p<0.05$). The proportion of transitional and secondary follicles was not affected by the culture system (ii). Bars denote mean + sem; n=5 for +meso, n=3 for P4 and - meso. Means with different letters are significantly different ($p<0.05$).

4.4.2 Ability of cultured germ cells to progress to the pachytene stage of prophase I.

Meiotic spreads were carried out on E13.5 ovaries that had been cultured for 6 days on an agar block. *In vivo* E18.5 ovaries were used as controls. Nuclei were counted and categorised into their respective stages of prophase I: leptotene, zygotene or pachytene. Homologous chromosomes in the oocyte nuclei from cultured ovaries were capable of progressing through leptotene and zygotene, to fully synapse at the pachytene stage of meiosis in an identical manner to E18.5 *in vivo* oocyte nuclei (Fig. 4.15). However, proportionally, fewer nuclei reached pachytene *in vitro* compared with *in vivo;* 23% *in vitro* compared with 50% *in vivo* (Fig. 4.16). The embryonic ovary culture system is therefore capable of supporting early meiosis of oocytes until the pachytene stage, although there was a slight delay when compared with the *in vivo* ovary.

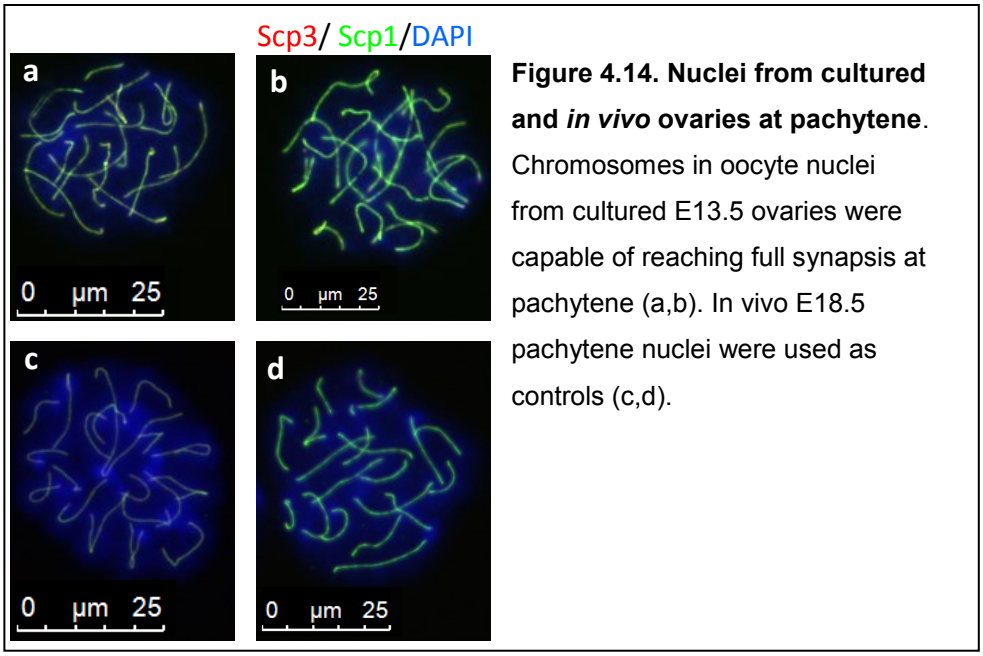


Figure 4.14. Nuclei from cultured and *in vivo* ovaries at pachytene. Chromosomes in oocyte nuclei from cultured E13.5 ovaries were capable of reaching full synapsis at pachytene (a,b). In vivo E18.5 pachytene nuclei were used as controls (c,d).

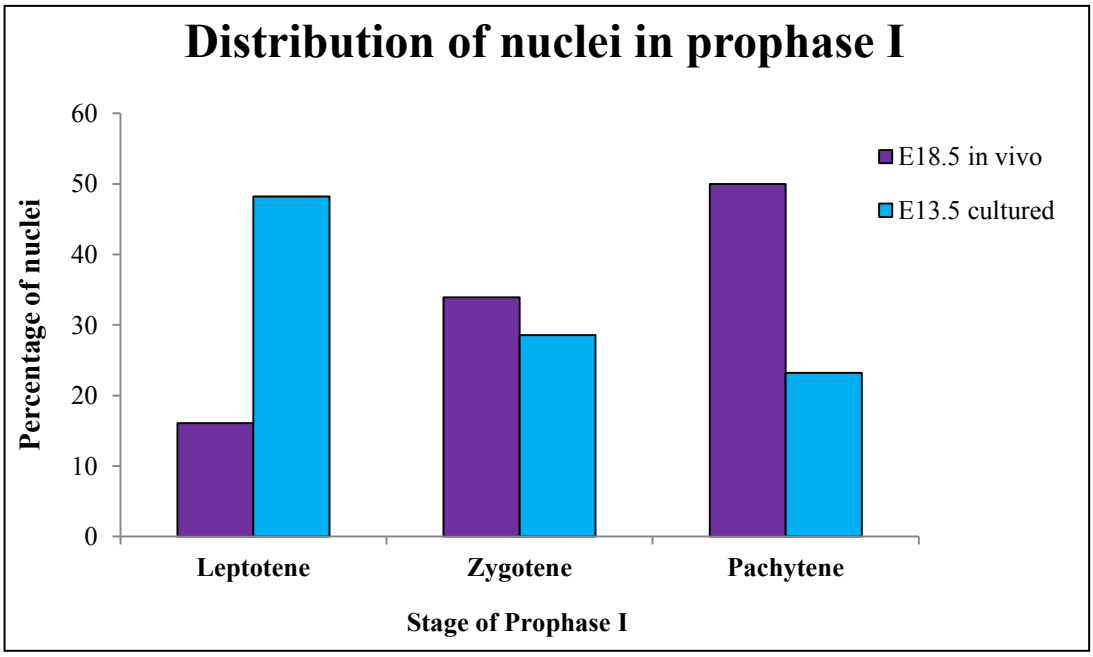
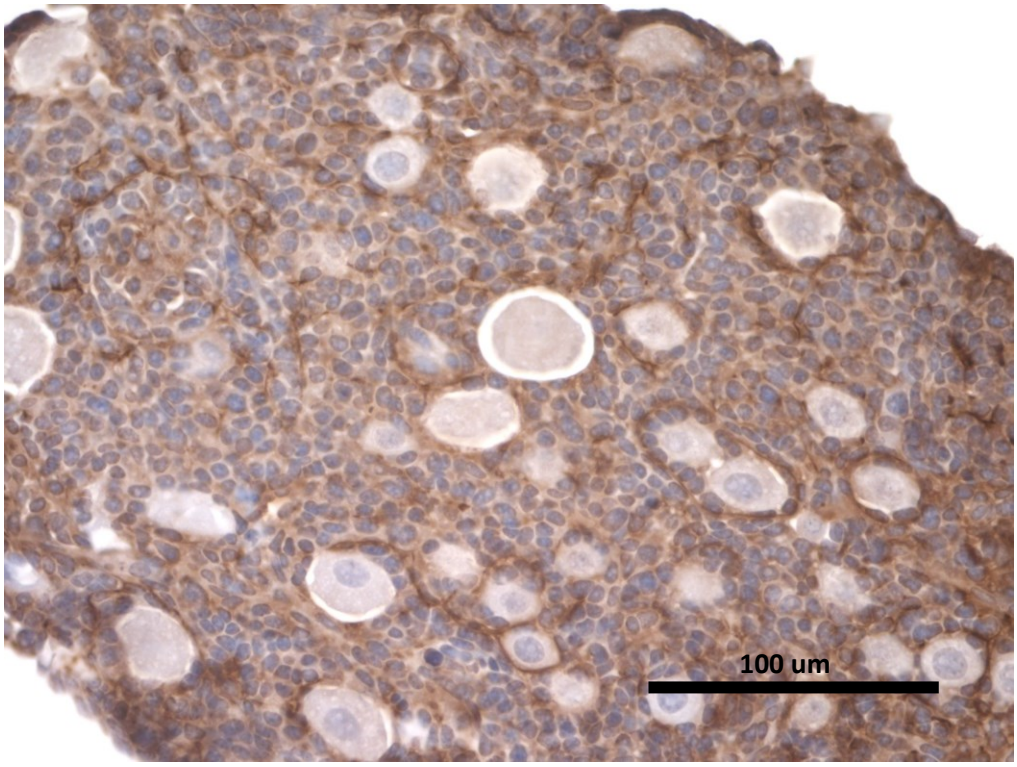


Figure 4.15. Distribution of cultured nuclei in prophase I of meiosis compared with in vivo. Nuclei from E13.5 cultured ovaries were capable of reaching the pachytene stage of meiosis, with 23% of nuclei reaching pachytene by 6th day of culture. However, this number was about half that observed *in vivo*, where 50% of nuclei had reached pachytene at E18.5. Roughly equal numbers of nuclei were observed in zygotene between *in vivo* and *in vitro.* Three times as many of the cultured nuclei were still in leptotene when compared with *in vivo* nuclei at E18.5.

4.4.3 Laminin-α1 protein expression in the cultured ovary

Laminin-α1 was selected as it is located within the basement membrane of follicles of all stages of development. Antibody detection proved problematic as intense staining was located in the BM of follicle, in the ECM of the stroma and the vasculature (Fig. 4.16).



4.16. Laminin immunostaining of an E13.5 ovary, cultured for 12 days.

4.4.4. Use of a biotin tracer to examine follicular architecture

Due to the problematic antbibody detection of laminin in cultured ovaries, a biotin tracer study was therefore tried to investigate follicular architecture and the establishment of basal lamina in cultured ovaries. Cultured E13.5 and P0 ovaries, as well as *in vivo* P4 ovaries, were incubated with a biotin tracer which was later visualised using a fluorescent antibody detection system. The biotin permeated freely into ovarian follicles at all stages, and was observed in stromal ECM, BM, as well as between GCs and in the ZP. While the follicles within the P4 uncultured ovaries showed clear structural organisation and basal membrane formation (Fig. 17a), cultured ovaries (P0 and E13.5) showed less follicular organisation, and fewer follicles appeared to have established a BM. Nevertheless, a large number of follicles within the cultured ovaries demonstrated appropriate formation of BM (Fig.4.17b,c). The stain intensity in the ZP of cultured oocytes appeared much greater than in that of *in vivo* ovaries. Furthermore, the ZP stain was much greater than that of the BM in cultured ovaries, making the visualisation of the BM more difficult.

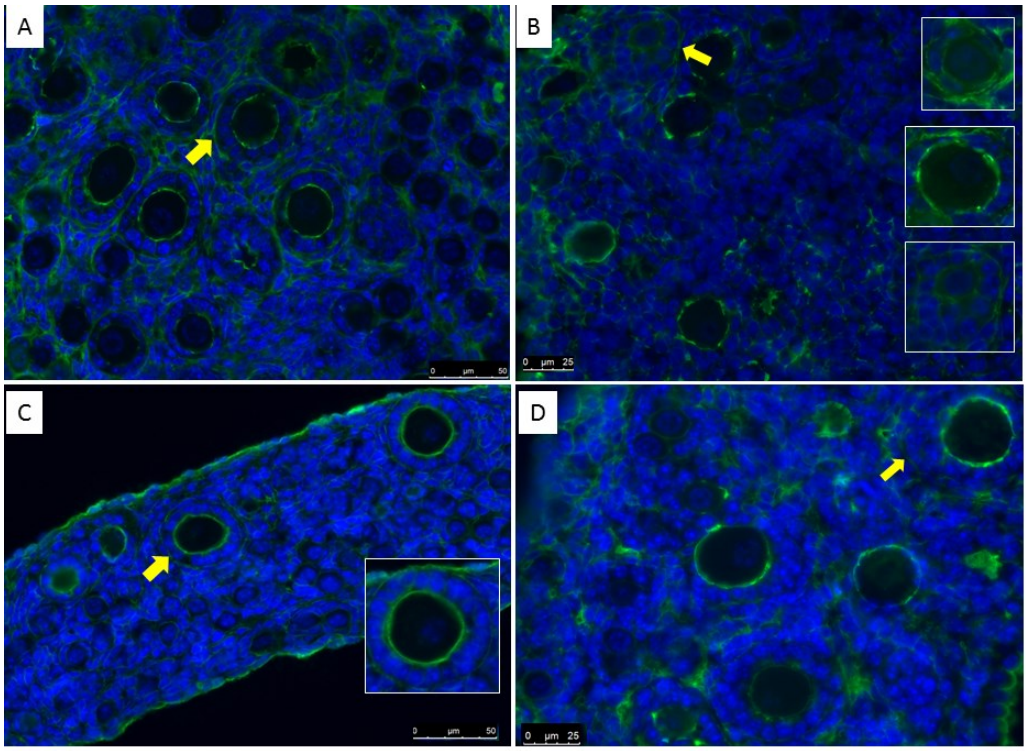


Figure 4.17. Biotin tracer study. Sections from a P4 *in vivo* ovary (a), cultured P0 (c) and E13.5 ovaries (b,d). Follicular organisation was less structured in cultured ovaries. Although the BM was a lot less defined in most E13.5 follicles, it was still visible in certain follicles ranging from the primordial to primary stage (see arrows). Follicles in P0 ovaries showed clear BM formation (c).

4.5 Discussion

A culture technique was established here whereby embryonic ovaries were successfully cultured from a pre-meiotic stage of ovary development, through prophase I of meiosis up-to dictyate, followed by a phase of follicle formation and growth. Various methodologies were attempted in order to optimise and bridge an existing embryonic ovary culture system with an existing neonatal ovary culture system. These included modifications to the culture medium (±βmercaptoethanol, inclusion of serum in the latter half of culture, varying the time-points for changing over to the simple medium), culture techniques (hanging droplet, membrane, agar) and inclusion or exclusion of the mesonephros prior to culture. Ovary health and morphology were examined histologically, chromosome spreads were carried out to investigate the progression of oocytes through meiosis, and biotin tracer studies were carried out to examine follicle architecture.

4.5.1. Optimisation of the early embryonic culture system

As the first germ cells in the mouse ovary enter meiosis around E13.5 (Peters, 1969, Peters, 1970), this was considered a suitable starting point for a culture system designed to encompass from meiotic entry of germ cells, through to meiotic arrest. Furthermore, these ovaries appeared to develop better in culture and produced healthier looking oocytes than ovaries from younger embryos (E12.5). Therefore, E13.5 ovaries were selected as a starting point. For the initial shorter cultures, several methods of culture techniques were attempted, including a hanging droplet, floating membrane and agar block culture; ovaries cultured on the agar block were able to better maintain their structural integrity and consistently appeared healthier than those cultured using the other methods.

Cultured ovaries are usually slightly developmentally delayed when compared with the *in vivo* ovary (Wang et al., 2013). Here, during the initial shorter culture (6 days), embryonic ovaries had developed from a pre-meiotic stage, to the rough equivalent stage of around E17.5-E18.5 in vivo. The oocytes within these cultured ovaries had become larger and contained nuclei that appeared to be in the dictyate stage of meiosis, looking morphologically very similar to in vivo germ cells.

4.5.2 Bridging the embryonic and neonatal culture systems

In an attempt to bridge the embryonic ovary culture with the neonatal ovary culture, and in order to allow ovaries to establish primordial and perhaps growing follicles, a longer embryonic ovary culture was carried out (12 days). As above, several different methodologies were attempted. Ovary health was found to be best when they were left on the agar throughout the culture period, with rich medium replaced by a simple medium on day 3 of culture. When the medium was changed at later time-points, granulosa-oocyte associations would not fully establish, and the BM would not form, although growing oocytes were observed. This suggests that optimal granulosa cell association might not be required for initial growth of oocytes.

It was speculated that the rich medium might potentially contain too many factors and hormones interfering with oocyte growth and follicle formation in later stages of culture, perhaps being required only for early meiosis and oocyte development. The rich medium was therefore changed over to the simple medium at a series of earlier time-points to investigate if follicle morphology would improve. The best method turned out to be when ovaries were kept in the rich medium for the shortest time period (3 days). The granulosa cell layers established better and a BM became visible around an increasing number of follicles.

4.5.3 Serum does not affect follicle growth in later stages of embryonic culture

Further attempts at improving the methodology of the culture system were carried out where serum was added back into culture medium for the final 3 days of culture, in an effort to 'push' some follicles beyond the primary follicles stage, although this proved unsuccessful. This was not surprising as *in vitro* conditions are associated with a delay in the primary to secondary follicle transition within cultured ovaries (Wang et al., 2013). However, considering that the ovaries still appear very healthy at the end of culture, it would be interesting to carry out longer cultures, to examine if some follicles might be capable of growing to the secondary follicle stage.

4.5.4 The presence of germ cell nests within cultured embryonic ovaries

Germ cell nests were observed fairly frequently in ovaries at the end of culture, where around 2-6 germ cells remained associated in small clusters, with few if any associated flattened granulosa cells. Germ cell nest breakdown usually occurs around the time of birth in the mouse. Germ cell nests are believed to be maintained during fetal development due to the high levels of maternal E2 which inhibits germ cell nest breakdown and protects oocytes from programmed cell death (Tingen et al., 2009). As the pups are born, the radical drop in the level of circulating E2 has therefore been suggested to lead to germ cell nest breakdown within the neonatal ovaries (Chen et al., 2007). One possibility is that the Phenol Red within the DMEM and α-MEM in the culture medium was having a weak estrogenic effect on the ovaries, as Phenol Red has been shown to bind to the estrogen receptor and elicit weak estrogenic effects in cultured tissues (Berthois et al., 1986). Therefore, for future experiments it might be possible to use phenol red-free DMEM and α-MEM, to investigate if this helps reduce the occurrence of germ cell nests at the end of culture.

4.5.5 The effect of culturing embryonic ovaries with or without the mesonephros.

Initially, the aim was to culture the ovaries without the mesonephros. This was carried out on the principle that the *in vitro* ovary was more likely to benefit from the absence of the mesonephros in the long term, given that it degenerates around E16.5 in the mouse *in vivo*. The mesonephros grows particularly well in culture, so it was also thought that it might spread out, overpower and interfere with ovary development, thereby hindering the ovary's ability to grow *in vitro.* It was then later speculated that the dissection process, whereby the mesonephros was removed, could potentially result in subtle damage to the ovary. This led to culturing embryonic ovaries with the mesonephros attached. These ovaries ended up appearing morphologically very healthy and with better established BM around the follicles. Suprisingly, the mesonephros did not take over or fuse with the ovary. It remained attached to one side of the ovary and appeared to be more beneficial to ovary development than had been expected, with follicle numbers and ratios slightly closer to those observed in the *in vivo* ovaries, than if the mesonephros was removed prior to culture. It is possible that this difference was due to the subtle damage that the

ovary sustained whilst dissecting away the mesonephros using insulin needles, resulting in a small loss of follicles. On the other hand, considering the evidence for a mesonephric contribution of stromal cells to the ovary during embryonic development (Upadhyay et al., 1979), it is possible that if the mesonephros is dissected away too early, fewer cells end up invading the ovaries resulting in fewer somatic cells to form associations with oocytes. Finally, it is also possible that the mesonephros supplies the ovary with factors, such as RA, that contribute to meiotic entry (although the effect of RA has been debated; Section 1.1.1.2), and therefore if removed early, it could disrupt meiotic entry. A study has previously been carried out where the mesonephros was dissected from the ovary after a few days in culture (Obata et al., 2002). This was also attempted, but was near impossible to do without damaging the ovary.

4.5.6 The effect of embryonic ovary culture on follicle numbers

Follicles in ovaries cultured on agar were counted and categorised according to follicle health and stage. The significant drop in follicle numbers in cultured ovaries compared with in vivo ovaries has been repeatedly reported in cultures of fetal ovaries (Tavendale et al., 1992, McLaren and Buehr, 1990). A likely possibility is that the culture system is not yet optimised enough to allow the majority of germ cells to survive meiosis and germ cell nest breakdown to form follicles, or that it results in a larger wave of apoptosis during germ cell nest breakdown than normal. Oxidative stress and free radical damage are potential triggers for cell atresia in the developing ovary (Tilly and Tilly, 1995), as local tissue damage during ovary dissection or the culture environment might increase oxidative stress. However, the inclusion of β-mercaptoethanol, a free radical scavenger, in the culture medium should limit this. This was somewhat supported by the lack of visible oocytes when ovaries were cultured without β-mercaptoethanol. It is, also possible that the culture system could be interfering with the end stage of germ cell proliferation, prior to meiotic entry, resulting in fewer oogonia entering meiosis in the first place. This is unlikely however, since germ cell proliferation ceases before meiotic entry (Peters and Crone, 1967, Okhubo et al., 1996). Nevertheless, it is possible that the beginning of culture might overlap with the tail-end of germ cell proliferation, as not all germ

cells enter meiosis synchronously. Another possibility is that the stromal environment in culture is less healthy or contains fewer stromal or pre-granulosa cells than in vivo. This could result in the ovary being less able to support oocyte growth and follicle formation, with potentially fewer pre-granulosa cells making associations with oocytes. Inappropriate granulosa-oocyte cell associations appears to be a recurring issue observed in cultured embryonic ovaries both in the cultures described here and in a previous experiment examining cultured embryonic ovaries (McLaren and Buehr, 1990). It would, therefore, be interesting to investigate the presence of proliferating germ cells in ovaries at the start of the culture, for example by examining the expression of Ki67 (proliferation marker) and VASA (germ cell marker) at E13.5 or by adding BrdU during the first day of culture. Furthermore, it might be possible to study the number of apoptotic germ cells during germ cell nest breakdown in cultured ovaries, which could be compared to the number of germ cells lost during germ cell nest breakdown in in vivo ovaries, to determine if it is during this stage that oocytes are lost during culture.

There is increasing evidence to suggest that PGCs require the presence of certain specific growth factors to avoid undergoing apoptosis. PGCs and early oocytes, are therefore, most likely dependent on growth-factors for survival. These include for example SCF/KL, bFGF and LIF, as well as insulin growth factor-I (IGF-I) and neurotropin 4/5 (Tilly, 2001, Morita et al., 1999, Lobascio et al., 2007). It would be interesting to investigate if the presence of these factors, in particular LIF and/or SCF, which promote primordial germ cell survival and proliferation *in vitro,* might reduce the number of germ cell lost during the culture period.

The observed reduction in the number of secondary follicles observed in the ovary was an expected result, as outlined above, neonatal mouse ovary cultures have been shown to delay the primary-to-secondary follicle transition (Wang et al., 2013).

4.5.7. Cultured ovaries had a normal geographical organisation of follicles

Histological examination showed that the geographical pattern of follicles within the cultured embryonic ovaries appeared normal, corresponding closely to the

organisation of follicles within the *in vivo* P4 mouse ovary. Non-growing primordial follicles were arranged around the outside of the ovary, with larger growing follicles located more centrally and by the medulla. This has been investigated previously where it was suggested that the growth pattern of follicles is established before birth, around E13.5, possibly related to the timing of meiotic entry (Byskov et al., 1997). A prior study using ovaries from before this time-point (E13.5) failed to develop a normal pattern of follicular organisation (Byskov et al., 1997). This further supported the argument for selecting E13.5 ovaries over E12.5 ones as starting material for this embryonic ovary culture system.

4.5.8 Progression of cultured oocytes through prophase I of meiosis

In order to investigate if homologous chromosomes within the cultured oocytes were capable of fully synapsing, meiotic chromosome spreads and SC visualisation were carried out on cultured oocytes at the time-point by which the majority of oocytes were expected to have progressed to the pachytene stage of prophase I. Many pachytene nuclei were observed from cultured oocytes, demonstrating that the culture system supports oocyte development to the pachytene stage of meiosis. Although similar number of oocytes had reached zygotene in both cultured and *in vivo* ovaries, fewer cultured oocytes had reached pachytene and a greater number were still at leptotene. It is therefore likely that the cultured oocytes were slightly delayed in comparison to the *in vivo* ovary. This result was not surprising given that a delay in ovary development is frequently observed *in vitro,* with follicle formation and growth often observed slightly later in cultured ovaries than at the equivalent age *in vivo* (Wang et al., 2013). The cultured ovaries in this study had been left in culture a day longer than their equivalent day *in vivo,* and were therefore expected to be roughly at the same meiotic stage as the *in vivo* ovary. This suggests that the meiotic delay is slightly greater than previously expected. It would be interesting to investigate if the culture system supports oocyte meiosis to dictyate, by examining whether the chromosomes are all paired using a chromosome spread technique (Henderson and Edwards, 1968), or by examining the expression of diplotene markers such as Msy2 (Gu et al., 1998), PAR6 (Wen et al., 2009) or TRP63 (Suh et al., 2006, Myers et al., 2014).

4.5.9. Investigation of the follicular architecture and basement membrane formation

P4 ovaries cultured briefly with a biotin tracer showed clear follicular organisation and BM formation in follicles of all stages. Cultured ovaries appeared less organised, and although the BM was visible for many follicles, it was less apparent than *in vivo*. This was partly due to the fact that the ZP in cultured oocytes stained more intensely than *in vivo* ovaries. The bright staining of ZP relative to the BM in cultured ovaries therefore made it more difficult to visualise the BM. It is possible that the *in vivo* ovaries had lower ZP stain because they were larger in volume than embryonic ovaries, meaning that the biotin tracer took longer to diffuse through the ovaries, resulting in less stain. It is also plausible that the ZP had hardened in culture. The ZP hardens naturally following fertilization in order to serve as a sperm barrier to avoid polyspermic fertilization, but prolonged exposure of oocytes to artificial culture conditions has been reported to induce zona hardening (De Vos and Van Steirteghem, 2000). If this occurred during the embryonic ovary culture, it might be a reason for the more concentrated ZP stain in cultured oocytes. Finally, it is also possible that the BM is less established in cultured ovaries, allowing more biotin to diffuse through the membrane and into the follicle resulting in a more intense ZP stain. Nevertheless, follicles within the cultured ovaries were capable of forming a BM.

4.6 Conclusion

This chapter details the development of a novel culture system that supports the growth and development embryonic mouse ovaries from a pre-meiotic stage, up-to a post-natal stage of follicle growth. It supports the development of germ cells through prophase I of meiosis to pachytene, albeit with a slight delay compared with *in vivo*, followed by the formation of primordial follicles and initiation of follicle growth. Although fewer follicles remain at the end of the 12 day culture period compared with the *in vivo* P4 ovary, the follicles are morphologically normal and healthy, and exist in comparable ratios to those *in vivo.*

4.7. Future directions

The next priority would be to investigate if the primordial follicles could progress to the secondary follicle stage during culture, by leaving the ovaries in the simple culture medium for longer periods. If successful, the ovaries could then potentially be disaggregated to carry out individual oocyte-cumulus cell complex cultures and extend the culture system further into antral follicle growth. Ideally, the individual cumulus-oocyte complexes could then be matured to a pre-ovulatory stage and 'ovulated' to complete meiosis I *in vitro.* It would also be important to carry out analysis into whether the oocytes are appropriately established in dictyate by carrying out chromosome spreads or immunohistochemistry. Although histological analysis of the chromosomes can give a good idea of the meiotic stage of a germ cell by, it is not sufficient to prove that they are appropriately established in dictyate.

Chapter 5.
Use of the embryonic ovary culture system for reproductive toxicity studies

5.1 Introduction

The testing of female reproductive toxicology is complex, where effects on both the pre- and post-natal ovary must be carefully considered. Although the majority of reproductive toxicology tests are currently performed *in vivo* (Sections 1.2 and 4.1), various ovary cultures have been established that have also been used in reproductive toxicity studies. These *in vitro* studies have been primarily carried out through academic research, consistently demonstrating the value of *in vitro* culture as potential preliminary investigations or secondary screening for potential reproductive toxicants (Iguchi et al., 1990, Chen et al., 2007, Devine et al., 2002a, Devine et al., 2004, Miller et al., 2005, Gupta et al., 2006, Wan et al., 2010, Inada et al., 2012, Zhou et al., 2008, Xu et al., 2002, Desmeules and Devine, 2006, Soleimani et al., 2011). The large majority of these *in vitro* studies have, however, only been conducted on the post-natal ovary. Very few pre-natal ovary cultures have been tried, with an even smaller proportion of these having been used to examine the effects of potential toxicants on the developing ovary. To date, no adequate *in vitro* model has been validated for use within the pharmaceutical industry or environmental regulatory agencies to pinpoint potential reproductive toxicants on the pre-natal ovary.

5.1.1 Etoposide

Etoposide (VP-16) is a chemotherapeutic agent used in the treatment of lung cancer, germ cell cancers, leukemia, neuroblastomas, Kaposi's sarcoma, and lymphomas (Hande, 1998, Gupta et al., 1987). The effective therapeutic dose of etoposide in adults ranges between 100-230 mg/m^2, which results in a plasma concentration of between 5-60 μg/ml (Hande et al., 1984).

5.1.1.1. Mechanisms of action

Etoposide is an alkylating agent that has been suggested to interfere with the ability of Topo II to re-ligate the nick in the DNA strand, resulting in increasing DNA fragmentation and consequent cell death (Chen et al., 1984, Ross et al., 1984, Gupta et al., 1987, Fortune and Osheroff, 2000) Another mechanism of action for etoposide has also been suggested, where it is thought to poison Topo II by increasing the

steady-state concentration of covalent DNA cleavage complexes within the Topo II enzyme, which turns Topo II into a physiological toxin that introduces high levels of single and double strand breaks (DSBs) into the DNA. The increasing amount of DSBs rises triggers a series of events, eventually resulting in apoptotic cell death (Hande, 1998).

5.1.1.2 Reproductive toxicity of etoposide

Around 1.5 million people were diagnosed with cancer in the year 2010. From this group, around 10% were younger than 45 and 1% younger than 20 (Jensen et al., 2011). Due to modern cancer therapy treatments, the overall 5-year survival rate has improved to nearly 80% for patients under 50 (Jensen et al., 2011). The side-effects of chemotherapy treatment, however, frequently involve premature ovarian insufficiency (POI) and loss of fertility (Fisher et al., 1979, Meirow et al., 1999, Wallace et al., 1989, Tauchmanova et al., 2002, Mackie et al., 1996, Familiari et al., 1993). When the increasing survival rate of cancer survivors is joined with the potential reproductive effects of chemotherapy treatments, this has resulted in cancer patients' rising concerns for future fertility. Fertility preservation has become an emerging field, but fertility preservation treatments are not always possible, or successful (Sonmezer and Oktay, 2004).

Etoposide treatment has been shown to induce ovarian effects in mice and hamsters, where the frequency of aneuploid oocytes and chromosome aberrations were increased following exposure (Mailhes and Marchetti, 1994, Tateno and Kamiguchi, 2001b). The evidence for the reproductive toxicity of etoposide in the human ovary has, however, been somewhat conflicting. A study investigating the fertility of women following etoposide exposure, demonstrated ovarian effects such as amenorrhea, anovulatory cycles or hypomenorrhea observed in a number of the female patients. Over half of these patients however, maintained normal ovulatory cycles, with a few successful pregnancies reported in several of the women who had previously been amenorrheic (Choo et al., 1985). Furthermore, a study investigating the fertility of an 18 year old woman who previously received a chemotherapeutic treatment that included etoposide, reported no effects on primordial follicle density

when compared with age-matched controls who had not received chemotherapy (Oktem and Oktay, 2007).

Etoposide has been prescribed to pregnant women where an aggressive malignancy posed a severe threat to the mother's life, such as lung cancer (Han et al., 2005, Kluetz and Edelman, 2008, Siepermann et al., 2012). The treatment was considered relatively safe for the fetus during the second and third trimesters and healthy babies were born following *in utero* etoposide treatment. Their development has been normal during the 6 years that the follow-up study has been carried out but no data has been reported on possible effects of etoposide on the reproductive system in these children, since none have reached puberty. As a result it is not known whether etoposide exposure resulted in adverse effects on their fertility, which may not become known until their adulthood. Furthermore, drugs targeting topoisomerases can increase genetic instability and therefore induce mutations within the developing germ cells. This could be transmitted to future generations but would not become apparent until the F2 generation (Baguley and Ferguson, 1998). It is therefore of high importance to investigate the potential effects of chemotherapeutic agents, such as etoposide, on the ovary, in particular the pre-natal ovary. An improved understanding of the mechanisms of mutagenesis is important not only to improve cancer therapy but also to fully understand the actions of topoisomerase targeted drugs, including etoposide (Baguley and Ferguson, 1998).

5.2 Aims

The primary aim of this work was to validate the novel mouse embryonic ovary culture by comparing the results following culture of embryonic ovaries with AZTC, with those from the in vivo study, where AZTC exposure had also occurred during embryonic development. Etoposide was selected as a relevant second study compound as it is also a Topo II inhibitor and has previously been prescribed to pregnant women.

5.3 Materials and Methods

5.3.1 Isolation of embryonic ovaries

CD-1 mouse breeding harems were set up as outlined in Section 4.3.2. Mouse embryos at E13.5 were collected, ovaries dissected and placed in 1xPBS (Section 4.3.5).

5.3.2 Preparation of culture plates and medium

2% agar was prepared and cut into 1cm^2 blocks using a sterilized blade (Section 4.3.3). The preparation of the rich PGC medium and the simple P0 medium was carried out as previously described (Rich medium: Section 4.3.4 and simple medium: Section 2.1.2). The agar block was placed in a 33mm petri dish, covered with the rich medium and placed in the incubator for 30 minutes to allow the PBS to diffuse out of the agar block (Section 4.3.4). The medium was then replaced with fresh rich medium, which was again allowed to incubate for 30 minutes to allow the media to equilibrate, prior to culture.

5.3.3 Assessment of Topo IIα expression in the *in vivo* and *in vitro* mouse ovary.

The expression of Topo IIα within the *in vivo* rat ovary was found to change from within the germ cells pre-natally, to the surrounding somatic and granulosa cells a few days after birth, as follicles began to form (Section 3.1.3). Therefore, to investigate if Topo IIα followed the same expression within the cultured ovaries, immunohistochemistry to detect Topo IIα was carried out on cultured embryonic ovaries. Embryonic ovaries (E13.5) were cultured for 12 days on agar as outlined above (5.3.2). Ovaries were collected at each day of culture, from day 1-12. Ovaries were washed in 1xPBS for 5 minutes, fixed in 70% buffered formalin and processed (Section 2.2). *In vivo* mouse ovaries were also collected from E13.5-PND6, so that the expression pattern of Topo IIα in culture could be compared with the *in vivo* ovary at the equivalent stages.

5.3.3.1 Immunohistochemistry for Topo IIα

Topo IIα was detected in the ovary using DAB as previously described (Section 2.4). The primary antibody used was a rabbit polyclonal anti-Topoisomerase IIα (Abcam,

ab52934) at a 1:200 dilution. The secondary antibody used was biotinylated goat anti-rabbit (Dako) antibody at a 1:200 dilution. Antibodies were diluted in a solution of non-immunised goat serum (Section 2.4.3). Topo IIα was detected with DAB (Section 2.4.6). Immunohistochemistry for Topo IIα in the *in vivo* mouse ovaries was carried during my second visit to AstraZeneca (Fig. 3.2.), where a pressure cooker was used for antigen retrieval and an automatic stainer was used for immunostaining. Immunohistochemistry for the cultured embryonic ovaries was carried out in Edinburgh following the same protocol as outlined previously (Section 2.4).

5.3.4 Embryonic ovary cultures with AZTC

The studies outlined in this chapter were carried out prior to the experiments where the ovary was found to consistently culture slightly better with the mesonephros (Sections 4.3.6.5 & 4.4.1.5). As a result, all cultures described here were carried out without the mesonephros. Due to time-constraints these cultures were not repeated with the mesonephros attached. E13.5 ovaries were cultured for 3 days with the rich medium and 9 days with the simple medium. AZTC was dissolved in dimethyl sulfoxide (DMSO). An initial dose response study was carried out where the medium was supplemented with AZTC at final concentrations of 1, 10, 100, 200 and 500 μM. Due to the very poor follicle morphology of ovaries cultured with 200 and 500 μM AZTC, the final concentrations of 1, 10 and 100 μM were chosen for the study. The medium was supplemented with either DMSO or AZTC for the first 6 days of culture and ovaries were moved to control culture on day 6 of culture (Fig. 5.1). A total 8 ovaries were counted for the control and 10 μM group, and 7 ovaries for the 1 and 100 μM groups, from 3 independent cultures.

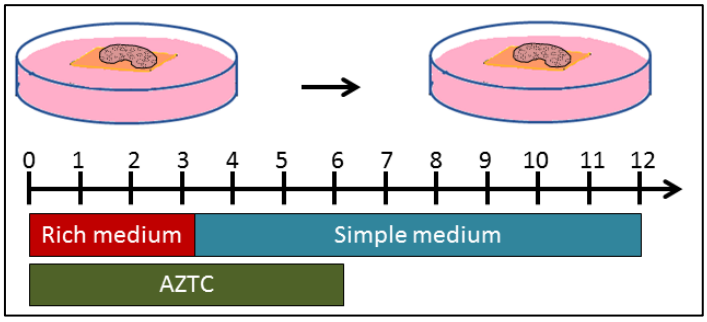


Figure 5.1. Outline of the methodology used for AZTC exposure in the embryonic ovary culture. Embryonic ovaries cultured 12 days on agar, in rich medium for 3 days and simple medium for 9 days. AZTC was added to the culture medium during the first 6 days of culture and replaced with control medium during the latter 6 days of culture.

5.3.4.1 Meiotic chromosome spreads on AZTC treated ovaries.

In order to assess the ability of cultured oocytes to progress through meiosis in the presence of AZTC, oocyte spreads were carried out on E13.5 ovaries that were cultured for 6 days with DMSO or 100 µM AZTC as previously described (Section 4.3.8). By the 6th day in culture the majority of oocytes were expected to be at pachytene if they developed as with *in vivo* control oocytes. Ovaries were first placed in a hypotonic extraction buffer for 15-30 minutes, followed by a sucrose solution where the ovaries were pierced with a needle to release the cells from the ovary. 10 µl of this cell suspension was picked up, placed on a pre-fixed slide and zig-zagged down the slide to spread out the cell suspension. The slides were placed in a humid chamber overnight and left air dry for 1-2 hours the following morning (Section 4.3.8). The slides were immunostained for Sycp1 and Sycp3 antibodies as outlined previously (Section 4.3.8.5) and visualised.

5.3.5 Embryonic ovary cultures with Etoposide

The ovarian effects of another Topo II inhibitor, etoposide, were investigated. This was carried out in order to investigate if the effects observed following etoposide exposure, which is known to inhibit mammalian Topo II, correlated with those of AZTC exposure.

Initially, a dose-response study was carried out on cultured neonatal (P0) CD1 mouse ovaries. The neonatal (P0) ovary culture was set up outlined previously (Section 2.1). Etoposide was dissolved in DMSO and the culture medium was supplemented with etoposide at the final concentrations of 25 ng, 250 µg or 25mg/ml for the whole duration of culture. This range of doses was selected to cover the plasma concentrations (5-60 µg/ml) reached in patients following etoposide treatment.

The embryonic ovary culture outlined above (Section 5.3.2) was repeated with etoposide at final concentrations of 50, 100 and 150 ng/ml (Sigma, E1383) during the inital 6 days of culture (Fig. 5.2). Ovaries were cultured for 12 days, for 3 days in rich medium followed by 9 days in simple medium (Section 4.3.6). A total of 6 ovaries were assessed for each treatment group from 2 independent cultures.

Similarly to above, these experiments were carried out prior to the methodology experiments where the ovary was found to consistently culture slightly better with the mesonephros (Sections 4.3.6.5 & 4.4.1.5), and therefore all cultures described here were carried out without the mesonephros.

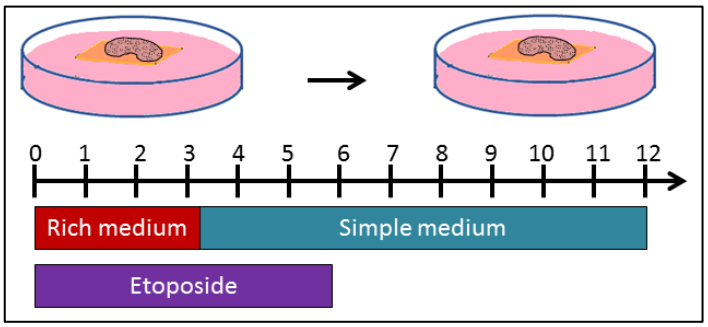


Figure 5.2. Outline of the methodology used for Etoposide exposure in the embryonic ovary culture. Embryonic ovaries cultured 12 days on agar, in rich medium for 3 days and simple medium for 9 days. Etoposide was added to the culture medium during the first 6 days of culture, followed by culture in control medium during the latter 6 days of culture.

5.3.6. Histological examination of cultured ovaries

E13.5 ovaries were cultured on an agar block for 12 days (3 days rich medium, 9 days simple medium, with AZTC or etoposide added during the first 6 days of culture). Ovaries were fixed in formalin, washed in 70% ethanol, embedded in agar, processed, embedded, cut and H&E stained as described in Section 2.2. Every 6th section from each ovary was photographed at x20 magnification. Follicles were counted blind as treatment and categorised as outlined previously (Section 2.3).

5.3.7. Detection of double-strand DNA breaks by γH2AX immunofluorescence in ovaries exposed to etoposide.

The expression of γH2AX was detected within etoposide treated ovaries collected from the initial neonatal dose-response culture as well as following the embryonic ovary culture. (Note: the immunofluorescent staining protocol on the neonatal etoposide-treated ovaries was carried out by Stephanie Morgan). Fluorescence was used to detect γH2AX using the Vector Mouse On Mouse kit (Vector, BMK-2202). This kit was selected to reduce the amount of background staining previously observed when conventional immunofluorescent staining was carried out. Slides were dewaxed and re-hydrated as described previously (Section 2.4.1), followed by a 30 minute incubation in 3% H_2O_2/90% methanol. Slides were washed in buffer and incubated for 30 minutes in a blocking solution (Vector labs, M.O.M. blocking solution), followed by a wash and a further 5 minute incubation in MO.M. diluent solution. The solution was tipped off and the slides were incubated with the primary antibody γH2AX (Abcam, 22551) diluted at 1:200 for 30 minutes at room temperature. Slides were washed and incubated with the biotinylated secondary antibody solution (M.O.M. kit) for 10 minutes. Slides were then washed and a solution containing streptavidin 488 fluorescent antibody (Alexafluor, Invitrogen UK, S11223) was applied at 1:200 dilution for 5 minutes, while the slides were kept in the dark. Finally, the slides were incubated for 20 minutes in a solution of DAPI counterstain (Invitrogen, D3571) diluted at 1:10000 in H_2O. Slides were washed and mounted using Vectashield (Vector, H-1400), coverslipped and photographed using a Leica A6000 fluorescent microscope.

5.3.8 Statistical Analysis

Graphpad Prism was used for all statistical analyses of follicle number and distribution between DMSO control ovaries and ovaries exposed to AZTC or Etoposide as outlined in Section 3.3.3.

5.4 Results

5.4.1 Expression pattern of Topo IIα in the mouse ovary *in vitro & in vivo*

Topo IIα expression was observed within the germ cells during pre-natal ovary development, but changed to the surrounding granulosa and somatic cells at birth (Fig. 5.3). A similar expression pattern was observed in the cultured ovaries, where during the first 6 days of culture, Topo IIα was expressed within the germ cells. Around day 6-7 of culture, as follicles began to form, Topo IIα expression became localised to the surrounding granulosa and stromal cells (Fig. 5.4). Cultured ovaries had more non-specific background staining than did *in vivo* ovaries. Fewer granulosa and stromal cells appeared to stain positive within the cultured ovaries than in the *in vivo* ones, but the expression pattern was, nonetheless, consistent between *in vitro* and *in vivo* ovaries (Fig. 5.5).

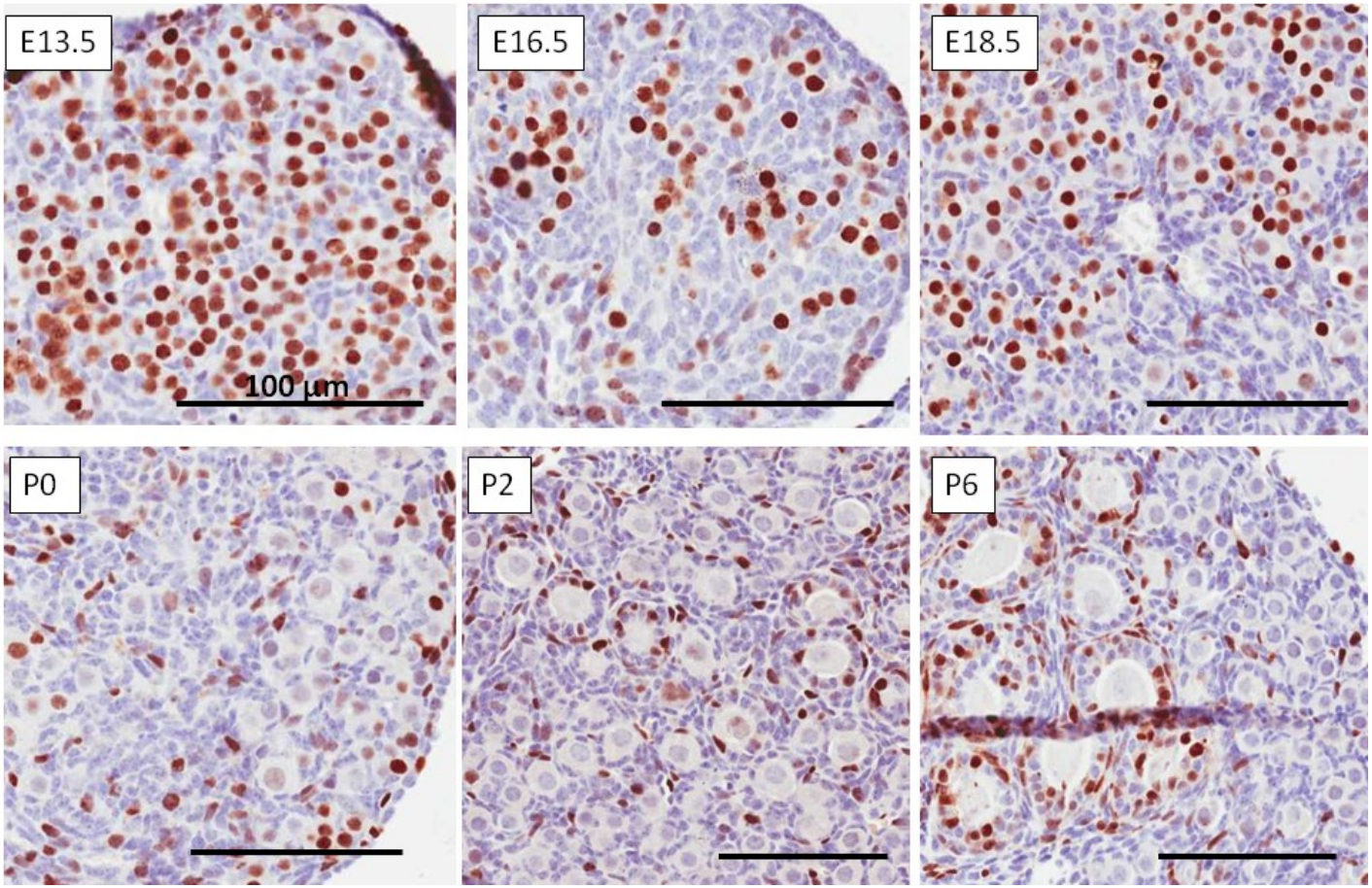


Figure 5.3. Expression of Topo IIα in the *in vivo* mouse ovary throughout pre- and post-natal development. Topo IIα was expressed within the germ cells pre-natally, but shortly after birth, as follicles began to form, its expression became localised to the surrounding granulosa and theca cells.

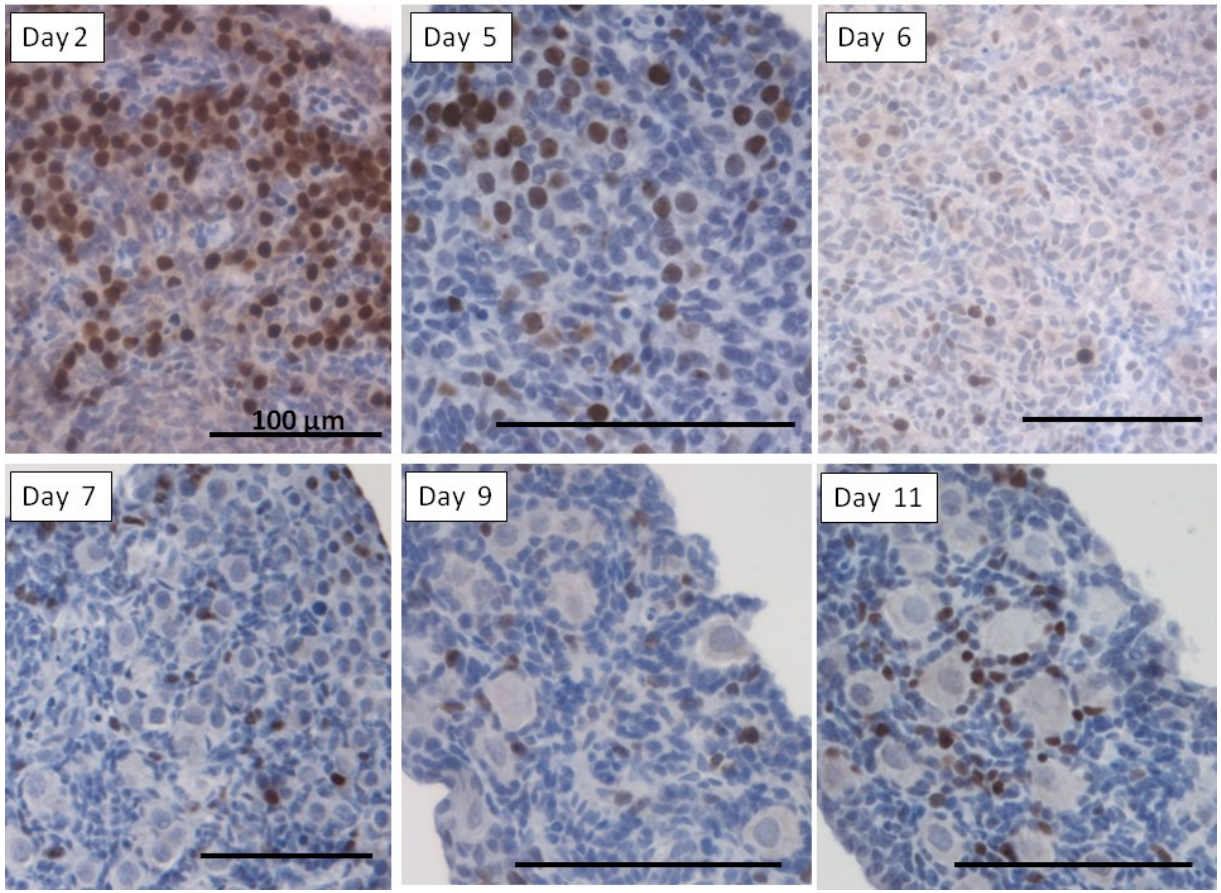


Figure 5.4. Expression of Topo IIα in the cultured embryonic mouse ovary throughout the 12 days of culture. Similarly to *in vivo,* Topo IIα was detected within the germ cells during the first 6 days of culture, prior to follicle formation. Around day 7 of culture, as follicles began to form, Topo II α was no longer detected in the oocytes but was expressed in the surrounding granulosa and stromal cells.

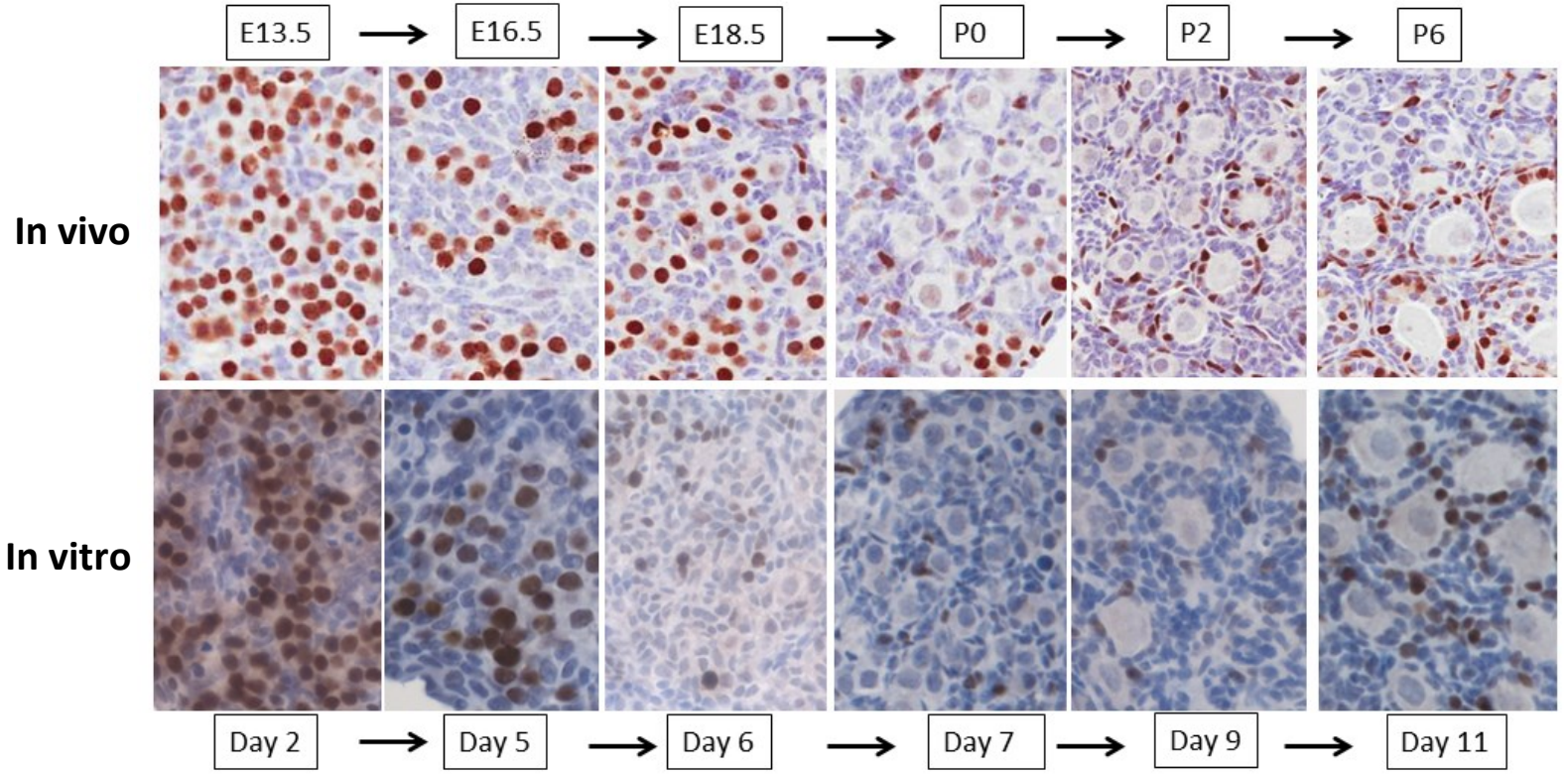


Figure 5.5. Comparison of the expression pattern of Topo IIα in the *in vivo* mouse ovary vs. the cultured embryonic mouse ovary. The same expression pattern is observed in the embryonic cultured ovary as is seen in the *in vivo* ovary. Topo IIα is expressed within the germ cells during the equivalent phase as 'pre-natal' ovary development, for the first 5-6 days of culture, until follicle formation. Once follicles begin to form, around birth *in vivo* and around days 6-7 of culture, Topo IIα expression is instead localised to the surrounding granulosa and stromal cells.

5.4.2 Effect of AZTC on the pre-natal ovary

E13.5 ovaries were cultured for six days initially with either DMSO or AZTC, followed by a further 6 days in control medium. A dose response study was carried out, where media contained final concentrations of AZTC at 1, 10, 100, 200 and 500 μM (Fig. 5.6). Ovaries cultured with 1, 10 and 100 μM concentrations of AZTC contained follicles ranging from primordial to primary stages, although by 100 μM the ovaries and follicles looked considerably less healthy. At the higher AZTC concentrations of 200 and 500 μM no follicles were observed and the ovaries appeared shrunken and atretic. The two top doses were therefore excluded from future studies.

E13.5 ovaries were cultured in the presence of DMSO or AZTC during the equivalent of the pre-natal phase development (n=8 for control and medium dose, n=7 for low and high doses). Ovaries exposed to the highest AZTC dose (100 μM) had significantly reduced follicle numbers ($p=0.0309$) whereas at the lower doses a slight, non-significant increase in follicle number was observed (Fig. 5.7). When follicles were distributed into the different follicle types a similar pattern was observed, with a slight non-significant increase in PMF numbers in low and medium doses, and with PMF numbers significantly dropping at the high dose ($p<0.001$) (Fig. 5.8i). AZTC had no significant effect on the number of TRNs, PRIM or SEC follicles. When the distribution of the percentage of follicles was examined, again a similar trend was observed for PMFs with increasing percentage of PMFs at the low and medium dose, with the percentage of PMFs significantly reduced at the high dose ($p<0.001$) (Fig. 5.8ii). Here, however, a significant increase in TRNS and PRIM percentages was also observed, with proportionally more TRNS and PRIM follicles remaining in high dose ovaries when compared with control ovaries.

When follicle health was examined, a slight dose-dependent increase in the number of unhealthy follicles was observed at the low and medium doses, but this was not significant. At the high dose, the number of unhealthy follicles was significantly reduced when compared with the medium dose ($p=0.0034$) (Fig. 5.9i). The percentage of unhealthy follicles within exposed ovaries compared with controls was not significantly affected ($p=0.767$) (Fig.5.9ii). When examining the distribution of unhealthy follicles, a significant increase in the number of unhealthy PMFs was observed at the low ($p<0.05$) and medium ($p<0.001$) doses, followed by a significant

reduction in the number of unhealthy PMFs at the highest dose when compared with low and medium doses. Other follicle types appeared un-affected by AZTC treatment (Fig. 5.10i). The percentage of unhealthy follicles within exposed ovaries were not significantly different to controls ($p=0.835$) (Fig. 5.10ii).

In order to determine the cell type responsible for the follicles being classified as unhealthy, and to determine which cell type was being targeted by AZTC, follicles were further classified into unhealthy due to the oocyte only, granulosa cells only, or both due to an unhealthy oocyte and granulosa cells. Nearly all the follicles within the AZTC treated embryonic ovaries were classified as unhealthy due to an unhealthy oocyte (Fig. 5.11).

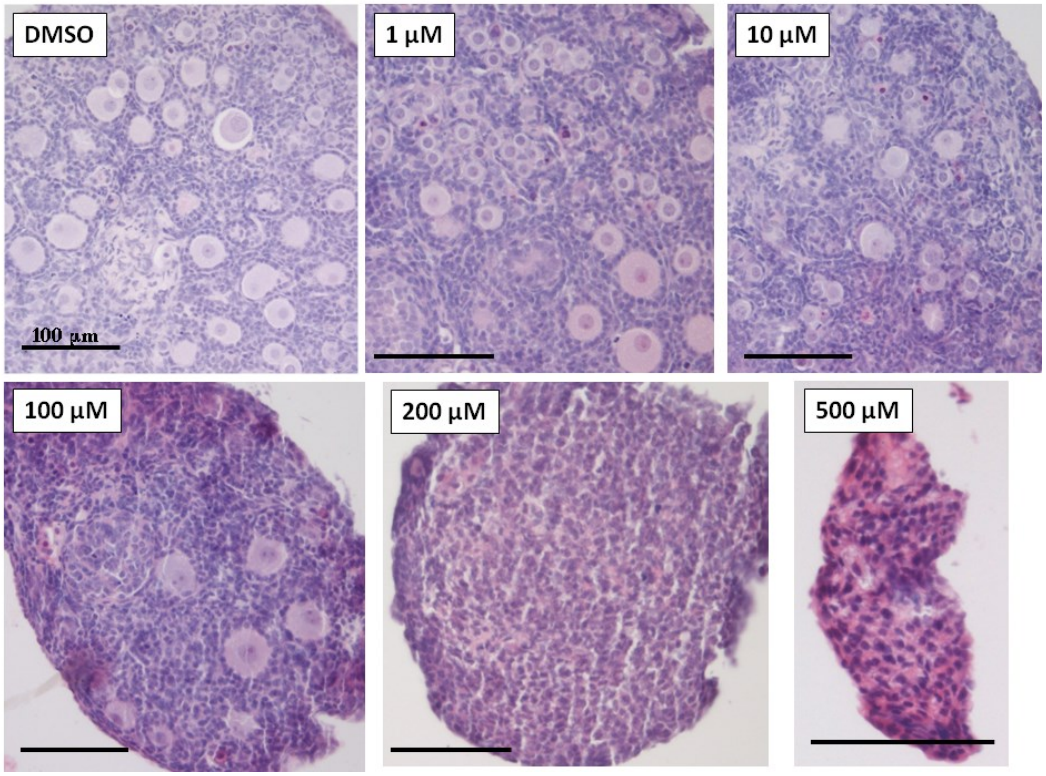


Figure 5.6. Dose response study with AZTC. Photomicrographs of ovary sections that had been treated with DMSO or with AZTC at final concentrations of 1, 10, 100, 200 or 500 µM. Ovaries treated with 200 and 500 µM AZTC appeared very atretic and shrunken with no remaining follicles. Ovaries at lower concentrations contained mostly healthy and some unhealthy follicles at various stages of development.

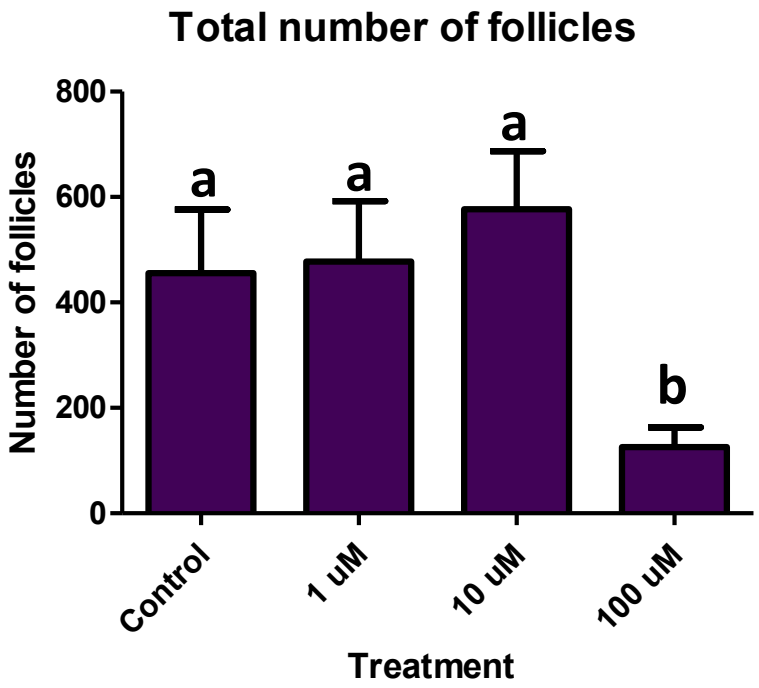


Figure 5.7. Total number of follicles within cultured ovaries. No effect of AZTC was observed on the follicle number within ovaries at the low and medium doses, although there appeared to be a slight non-significant increase in follicle numbers up-to the medium dose. A significant reduction in follicles had occurred within ovaries exposed to the high dose of AZTC after 12 days of culture ($p=0.0309$). Bars denote mean + sem; n=8 for control and 10 μM, n=7 for 1 and 100 μM. Means with different letters are significantly different ($p<0.05$).

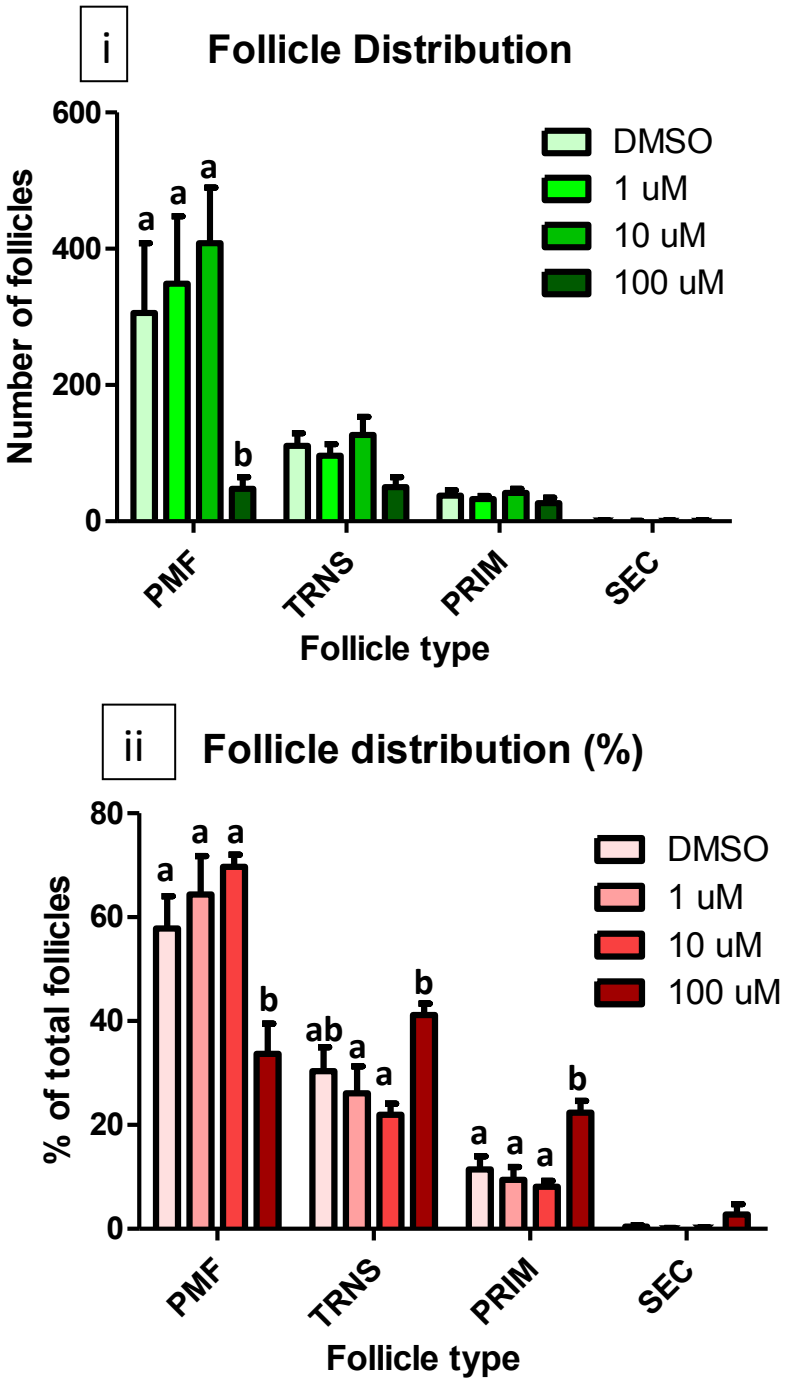


Figure 5.8. Distribution of follicle numbers (i) and percentages (ii) within embryonic ovaries cultured with AZTC. A slight non-significant increase in the number of primordial follicles was observed in the low and medium dose ovaries, although this number significantly dropped at the high dose ($p<0.001$) (i). A similar effect is observed when the percentages of primordial follicles were examined, with a significant increase in the percentages of transitional follicles (low vs. high dose: $p<0.05$; medium vs high dose: $p<0.01$) and PRIM follicles ($p<0.05$) was also observed at the high dose ((ii). Bars denote mean + sem; n=8 for control and 10 µM, n=7 for 1 and 100 µM. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different ($p<0.05$).

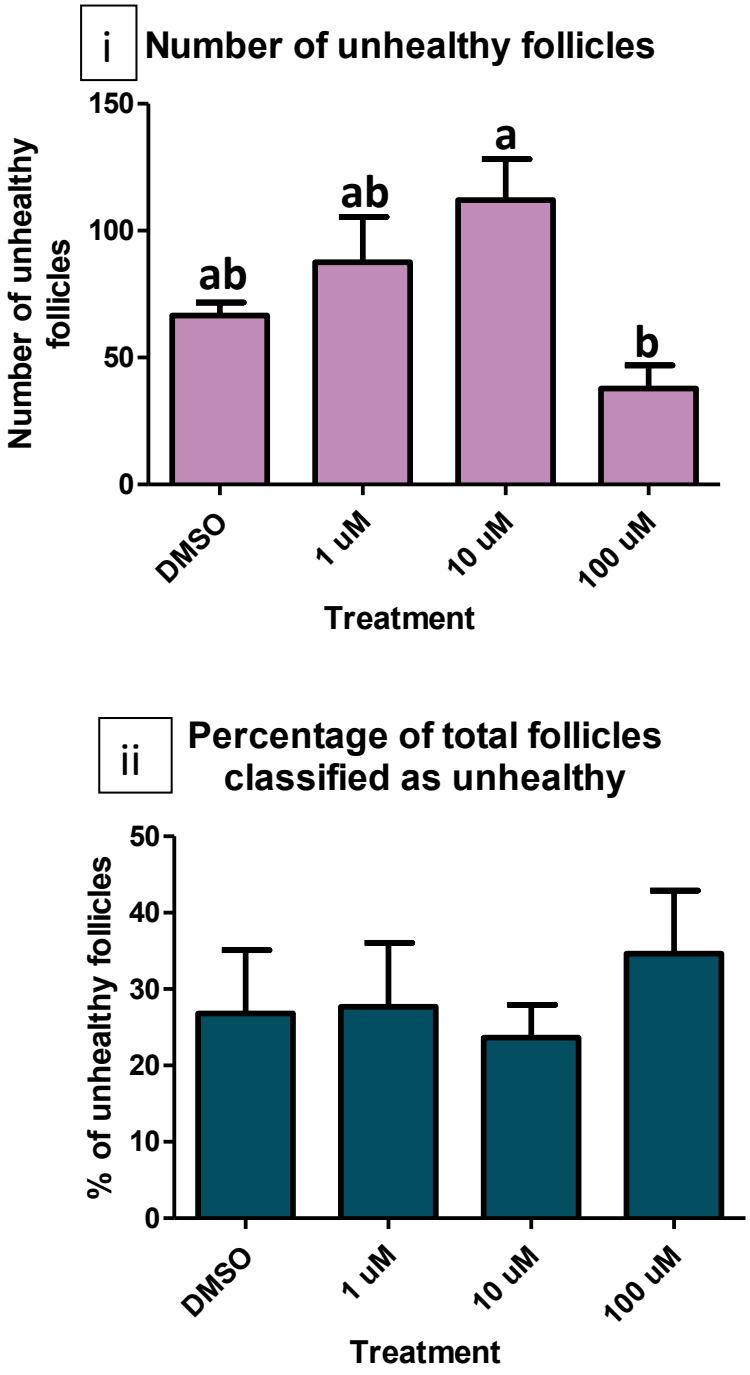


Figure 5.9. Follicle health within AZTC exposed ovaries. A slight non-significant, yet dose dependent increase was observed in the number of unhealthy follicles at the low and medium doses of AZTC, with a significant drop in the number of unhealthy follicles at the high dose when compared with the medium dose ($p=0.0034$) (i). No effect was observed on the percentage of unhealthy follicles between treated and control ovaries ($p=0.767$) (ii). Bars denote mean + sem; n=8 for control and 10 µM, n=7 for 1 and 100 µM. Means with different letters are significantly different ($p<0.05$).

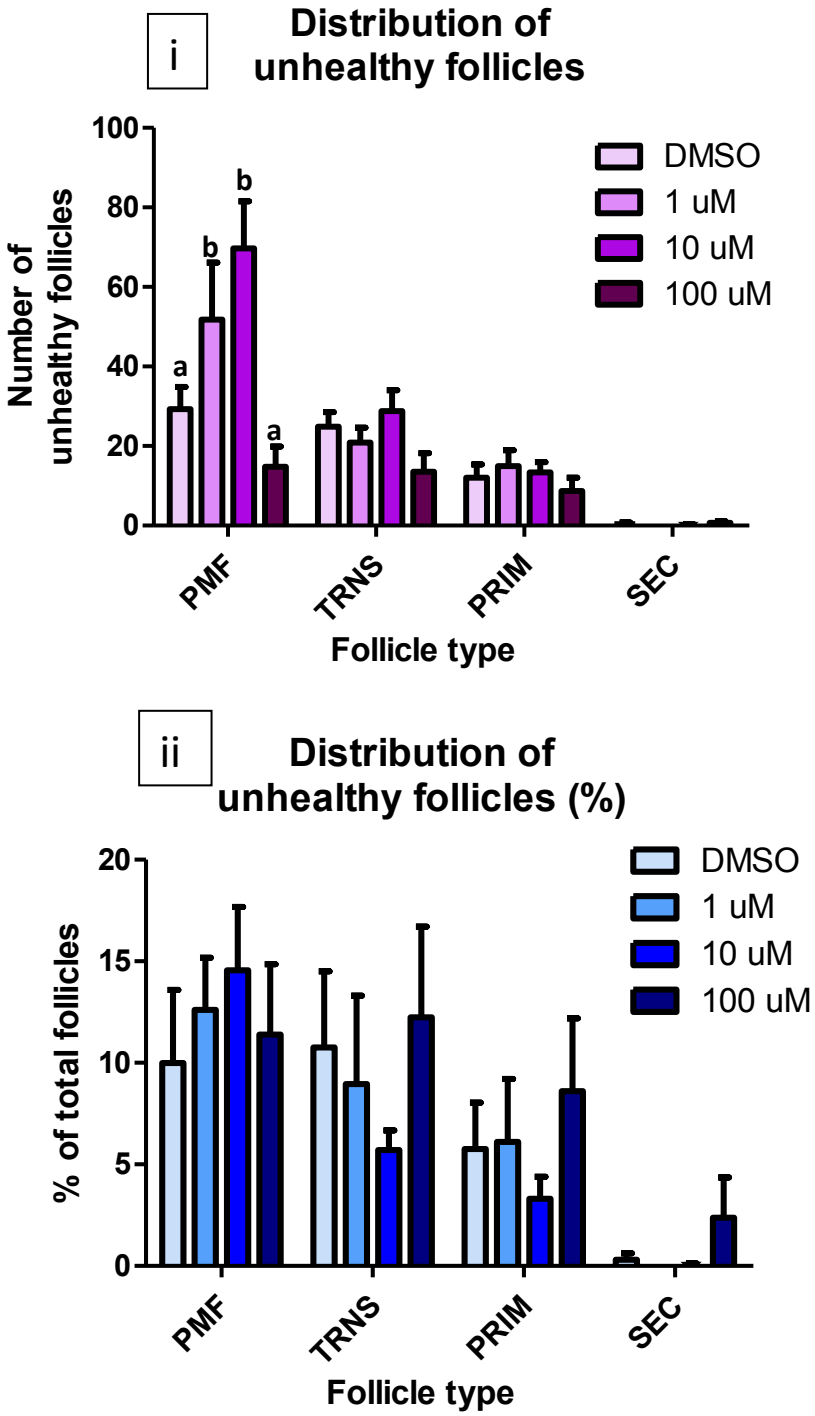


Figure 5.10. Distribution of unhealthy follicles within AZTC-treated ovaries. A significant dose-dependent increase in the number of unhealthy PMFs was observed up-to the medium dose, after which the number dropped to below that of control ovaries. No effect of AZTC was observed on other follicles types (i). When the percentages of unhealthy follicle types was examined, no significant differences were observed between control and treated ovaries ($p=0.835$) (ii). Bars denote mean + sem; n=8 for control and 10 µM, n=7 for 1 and 100 µM. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. . Means with different letters are significantly different ($p<0.05$).

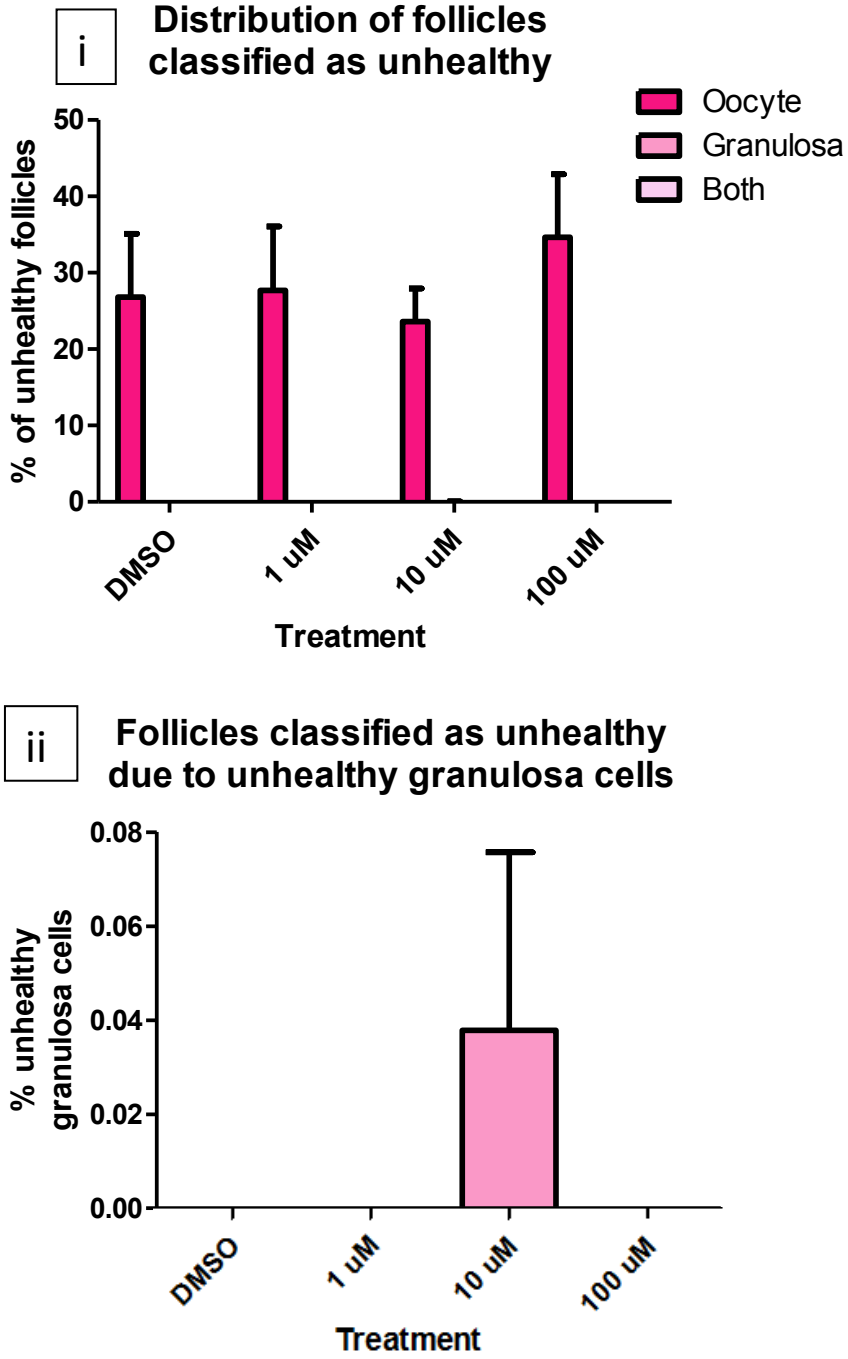


Figure 5.11. Distribution of follicles classified as unhealthy due to an unhealthy oocyte, granulosa cells or both. Unhealthy follicles were further classified into damaged cell types, with the vast majority of follicles being classified as morphologically unhealthy due to an unhealthy oocyte (i). No follicles were classified as being unhealthy due to both unhealthy oocytes and granulosa cells, and less than 0.1% of follicles were classified as unhealthy due to unhealthy granulosa cells at the medium dose of AZTC (ii). Bars denote mean + sem; n=8 for control and 10 μM, n=7 for 1 and 100 μM.

5.4.3 Meiotic chromosome spreads on AZTC treated ovaries.

In order to investigate if AZTC interfered with the ability of oocytes to progress through meiosis to the pachytene stage, meiotic chromosome spreads were carried out and the SC was visualised by immunofluorescence. Oocyte nuclei were obtained from embryonic ovaries that had been cultured for 6 days in the presence of 100 μM AZTC. Unfortunately, a low yield of oocytes made it difficult to carry out any further analysis into the proportion of meiotic stages within the treated ovaries (leptotene, zygotene or pachytene). Despite this, several pachytene nuclei were observed within the treated ovaries, appearing morphologically very similar to those observed in control ovaries (Fig. 5.12).

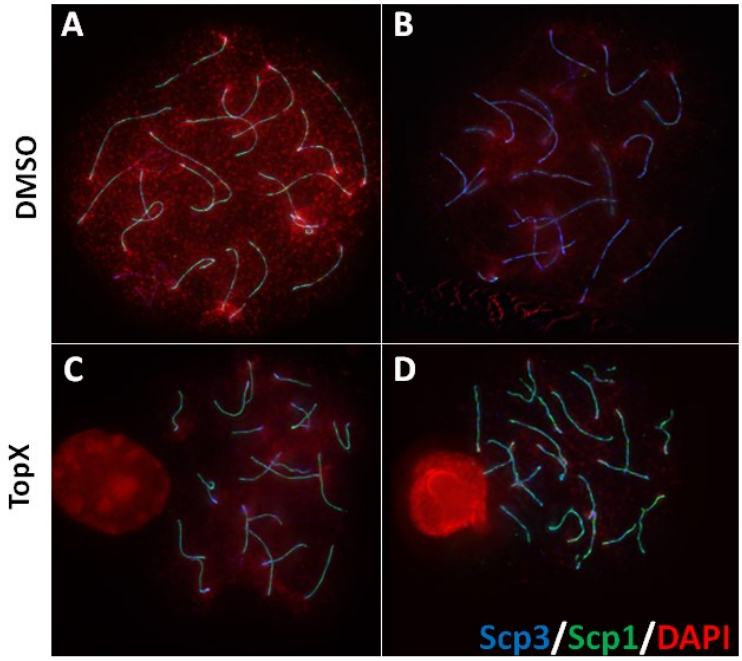


Figure 5.12. Meiotic chromosome spreads on control (A, B) and AZTC treated (C,D) ovaries. The chromosomes were capable of fully synapsing in the presence of AZTC (C,D) in a near-identical manner to control oocytes (A,B).

5.4.4 Effect of Etoposide on the pre-natal ovary

5.4.4.1. Dose response study

An initial dose-response study was carried out on cultured neonatal ovaries (CD1) to assess the appropriate doses of etoposide to be used, with final concentrations of etoposide in culture of 25, 250 ng/ml and 25 μg/ml. Ovaries treated with the lowest dose (25 ng/ml) appeared fairly healthy with varying stages of follicles and the occasional unhealthy granulosa cell (Fig. 5.13a, black arrows). Ovaries treated with the medium dose (250 ng/ml) appeared very unhealthy, with atretic oocytes and striated stroma, whereas ovaries receiving the highest dose (25 μg/ml) contained severely unhealthy stroma with no remaining follicles (Fig. 5.13d). Etoposide concentrations of 50, 100 and 150 ng/ml were therefore chosen for future experiments (n=9 for DMSO control, n=6 for 50 ng/ml, n=5 for 100 ng/ml and n=8 for 150 ng/ml). These doses were selected as they are lower than the doses that resulted in a complete destruction of oocytes but are also far lower the range of serum concentration measured following etoposide treatment (5-60 μg/ml).

Pre-natal *in vitro* exposure to etoposide resulted in a significant decrease in follicle numbers when compared with DMSO control ($p<0.01$) ovaries (Fig. 5.14). When the follicles were classified into their follicle types it became clear that the reduction in follicle numbers was due to a dose-dependent decrease in the number of primordial ($p=0.0085$) and transitional ($p=0.0041$) follicles with increasing dose of etoposide. No effect was observed on primary or secondary follicle numbers (Fig. 5.15i). When the percentages of each follicle type was examined, while no significant effect was observed on the proportion of primordial follicles within exposed ovaries, a significant reduction was observed in the proportion of transitional follicles at the highest dose when compared with control ($p<0.01$), low ($p<0.05$) and medium ($p<0.05$) dose ovaries (Fig. 5.15ii). A non-significant trend was also observed where the proportion of primary follicles increased slightly with increasing etoposide dose (Fig. 5.15ii). When analysing follicle health within etoposide exposed ovaries, it became evident that the number of unhealthy follicles within treated ovaries did not follow a normal dose-response curve. A slight non-significant increase in unhealthy follicle number was observed in low dose ovaries, with no effect observed at the middle dose. A significant reduction in unhealthy follicle numbers was observed however when compared with low-dose ovaries and DMSO controls ($p=0.005$, Fig. 5.16i). When the unhealthy follicles were classified into

their follicle types, a similar effect was seen as in Fig. 5.16i, where a non-significant increase was observed in the number of unhealthy PMFs in low dose ovaries, after which the number dropped back to the equivalent to that of control ovaries. A significant increase was observed in unhealthy PMF numbers from low to high dose ovaries ($p<0.01$). The number of TRNs follicles was also significantly reduced at the top dose when compared with controls ($p<0.01$ and middle ($p<0.01$) dose ovaries (Fig. 5.17i). A significant increase in the percentage of unhealthy follicles within ovaries exposed to the top dose when compared with controls ($p<0.05$) (Fig. 5.16ii). When the percentage of unhealthy follicle types was examined however, no significant differences were observed between control and treated ovaries (Fig. 17ii).

In order to determine the cell type responsible for the follicles being classified as unhealthy, and to determine which cell type was being targeted by etoposide, follicles were further classified into unhealthy due to the oocyte only, granulosa cells only, or both due to an unhealthy oocyte and granulosa cells. Nearly all the follicles within the etoposide treated embryonic ovaries were classified as unhealthy due to an unhealthy oocyte (Fig. 5.18).

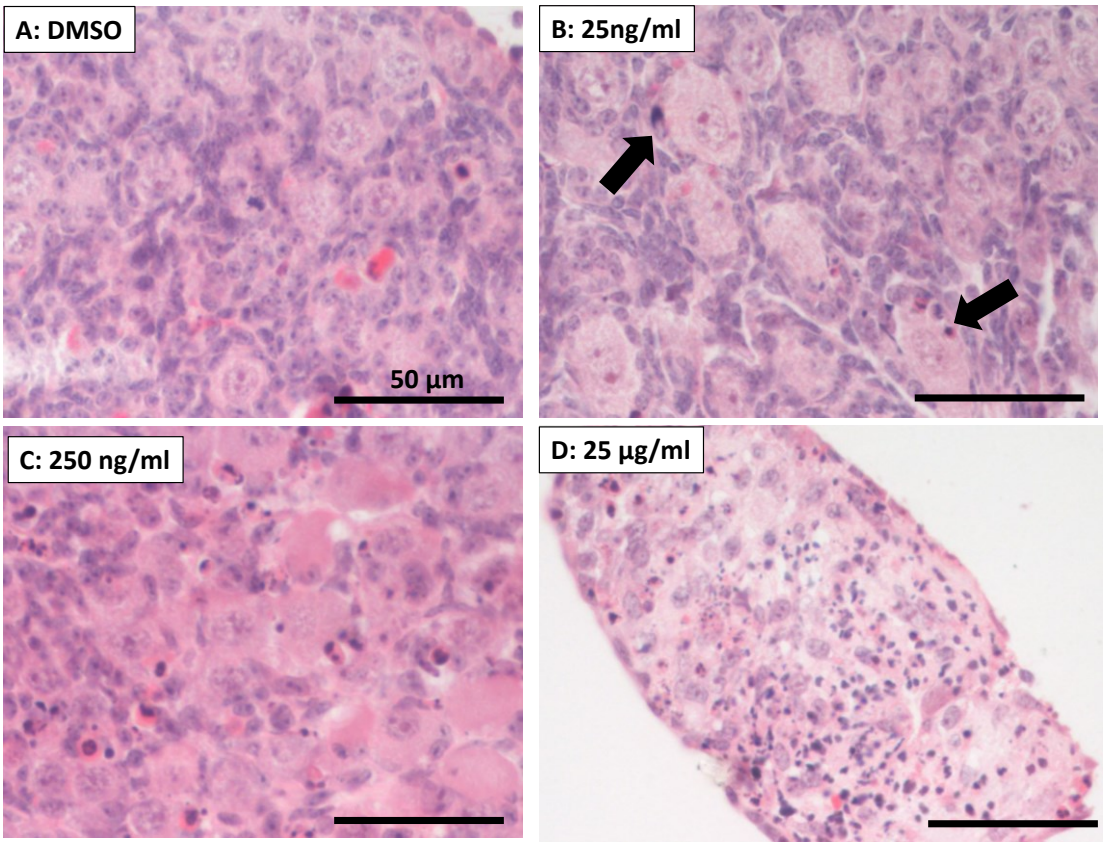


Figure 5.13. Dose response study for etoposide. Neonatal ovaries cultured either with DMSO (a) or etoposide at concentrations of 25 (b), 250 ng/ml (c) or 25 µg/ml (d). At the lowest dose, follicles remained fairly healthy with some unhealthy granulosa cells visible (b, black arrows), whereas middle dose ovaries appeared very unhealthy with all the oocytes appearing atretic and high dose ovaries were completely striated and atretic, with no follicles remaining.

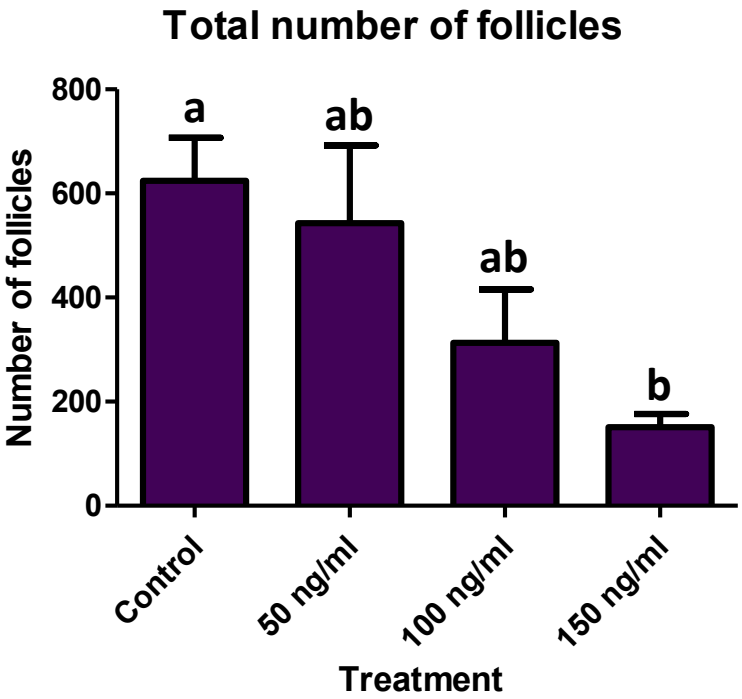


Figure 5.14. Follicle numbers within etoposide treated ovaries. Ovaries exposed to the high dose of etoposide had significantly reduced follicle numbers when compared with control ($p<0.01$) ovaries. Bars denote mean + sem; n=9 for DMSO control, n=6 for 50 ng/ml, n=5 for 100 ng/ml and n=8 for 150 ng/ml. Means with different letters are significantly different ($p<0.05$).

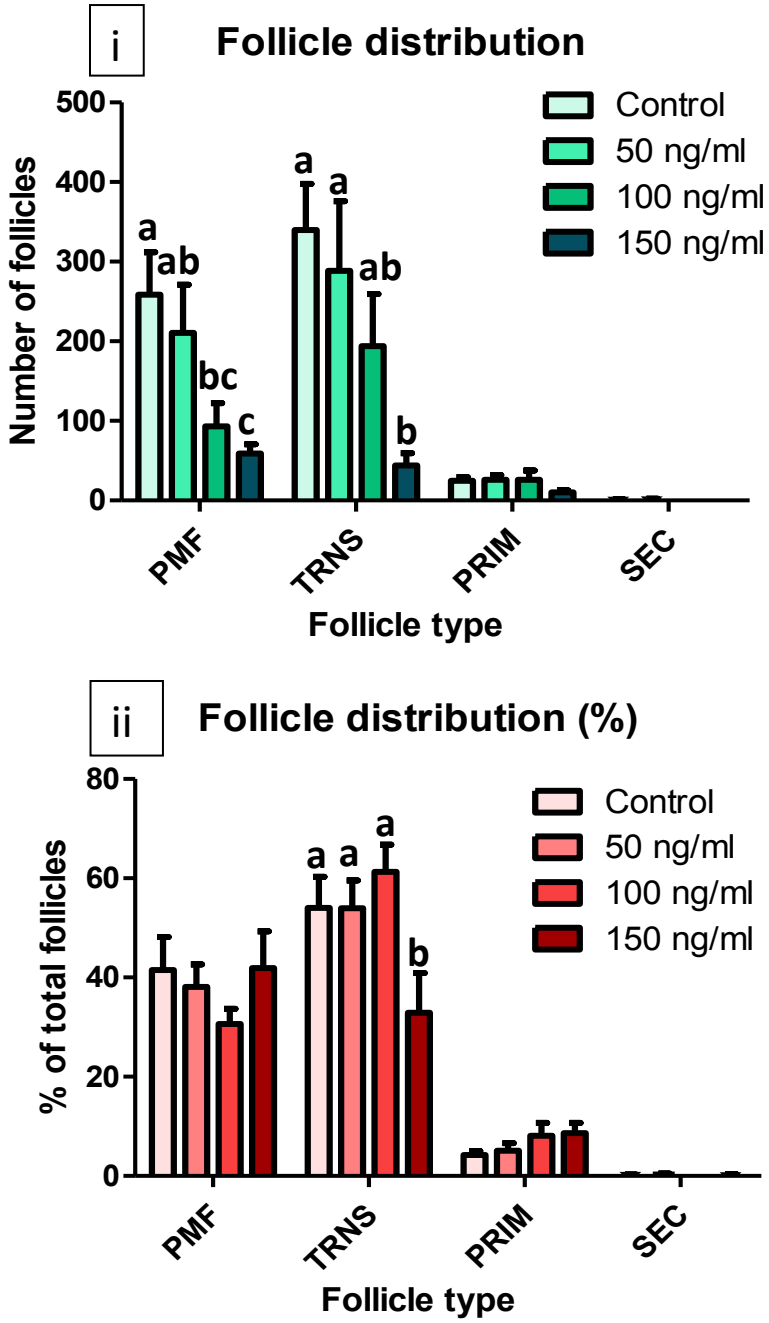


Figure 5.15. Distribution of follicles within ovaries exposed to etoposide. There was a significant dose-dependent reduction in the number of primordial (p=0.0085) and transitional (p=0.0012) follicles with increasing dose of etoposide. No effect was observed on primary or secondary follicle numbers (i). When the percentages of each follicle type was examined, no significant effect was observed on the proportion of primordial follicles within exposed ovaries, but a significant reduction was observed in the proportion of transitional follicles at the highest dose when compared with control ($p<0.01$), low ($p<0.05$) and medium ($p<0.05$) dose ovaries (ii). Bars denote mean + sem; n=9 for DMSO control, n=6 for 50 ng/ml, n=5 for 100 ng/ml and n=8 for 150 ng/ml. PMF: primordial, TRNs: transitional, PRIM: primary, SEC; secondary. Means with different letters are significantly different ($p<0.05$).

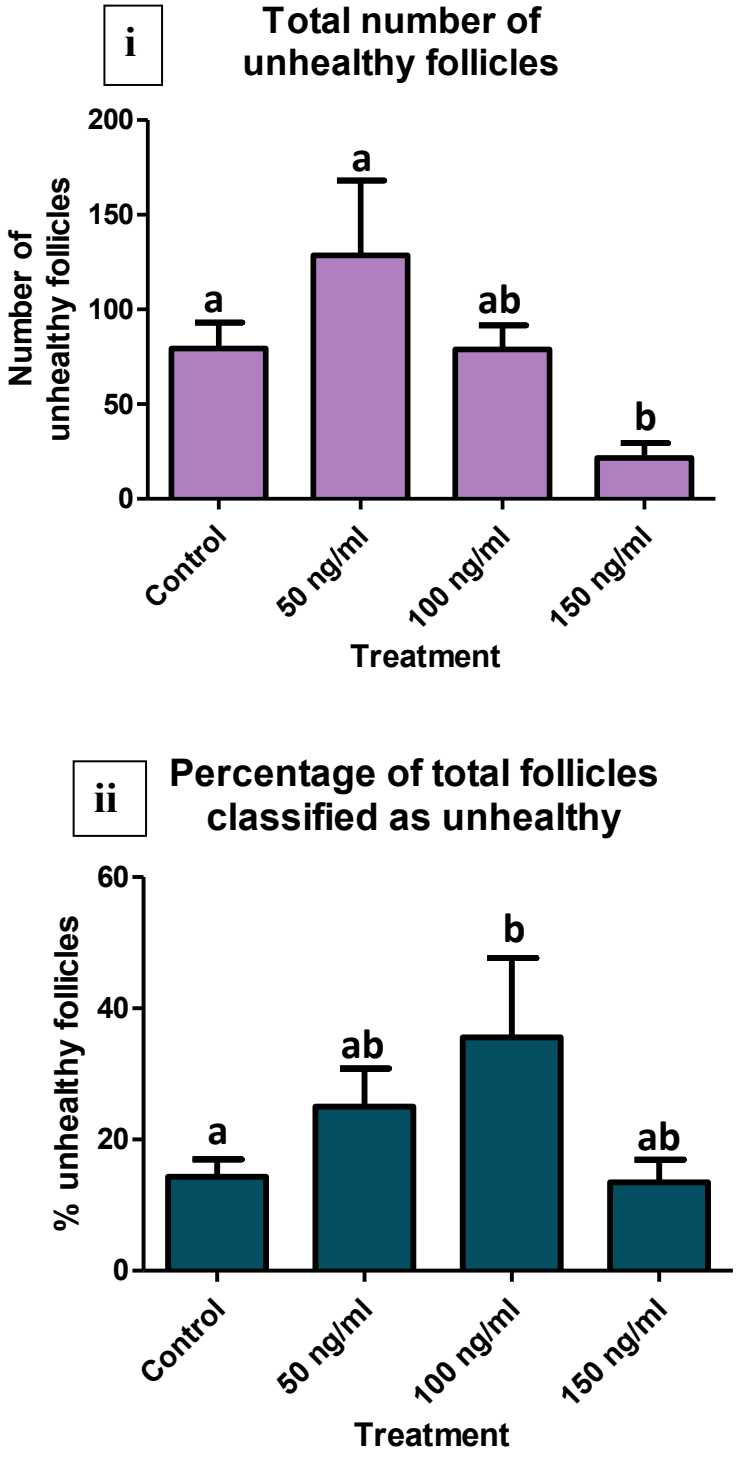


Figure 5.16. Follicle health within ovaries exposed to etoposide. A slight non-significant increase in unhealthy follicle number was observed in low dose ovaries, with a significant reduction at the high dose (i) ($p=0.005$). A significant reduction in unhealthy follicle numbers was obeserved when compared with low-dose ovaries ($p<0.01$) but not DMSO controls (i). There was also a significant increase in the percentage of unhealthy follicles within ovaries exposed to the 100 ng/ml dose when compared with controls ($p=0.0449$) (ii). Bars denote mean + sem; n=9 for DMSO control, n=6 for 50 ng/ml, n=5 for 100 ng/ml and n=8 for 150 ng/ml. Means with different letters are significantly different ($p<0.05$).

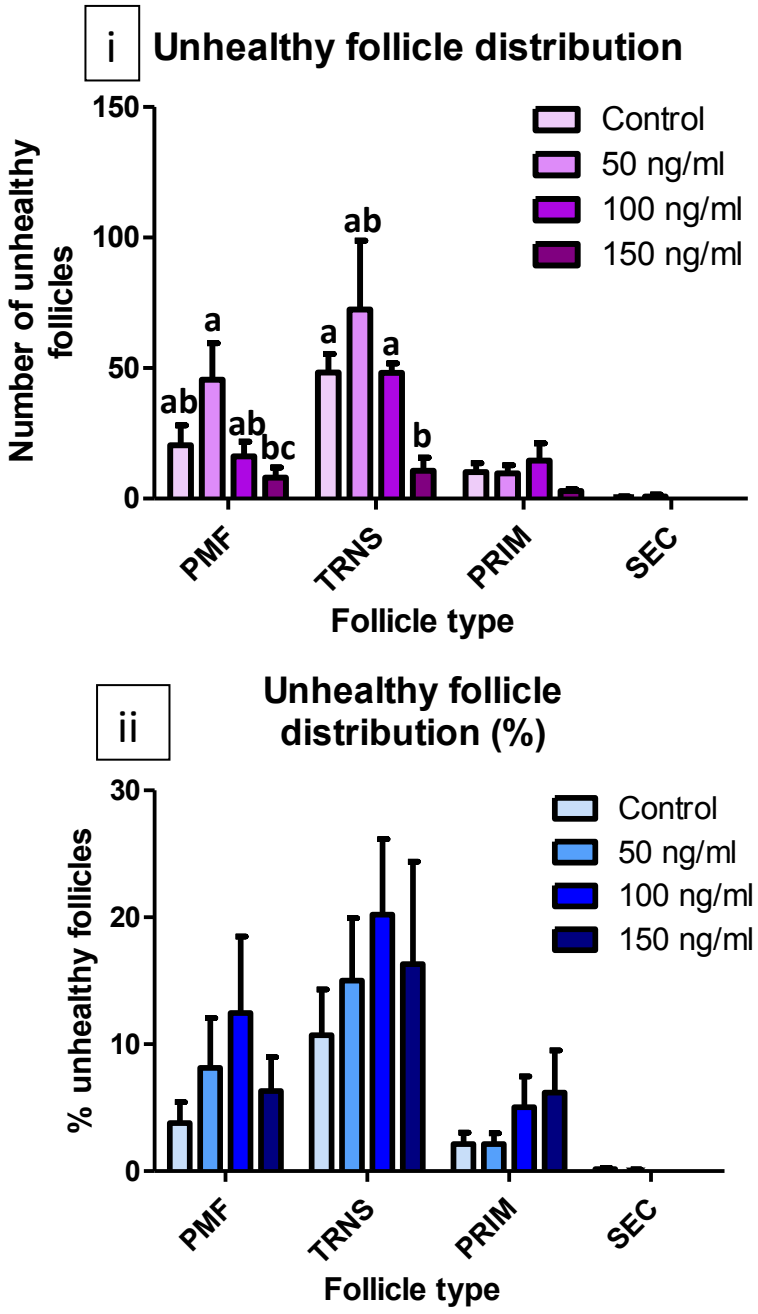


Figure 5.17. Distribution of unhealthy follicles within etoposide treated ovaries. A non-significant increase was observed in the number of unhealthy PMFs at the low dose, after which the number dropped back to the equivalent to that of control ovaries. A significant difference was observed in unhealthy primordial follicle numbers between low and high dose ovaries ($p<0.01$). The number of transitional follicles was also significantly reduced when compared with controls ($p<0.01$), and middle ($p<0.01$) dose ovaries (i). When the percentage of unhealthy follicle types was examined, no significant differences were observed between control and treated ovaries (ii). Bars denote mean + sem; n=8 for control and 10 µM, n=7 for 1 and 100 µM. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different ($p<0.05$).

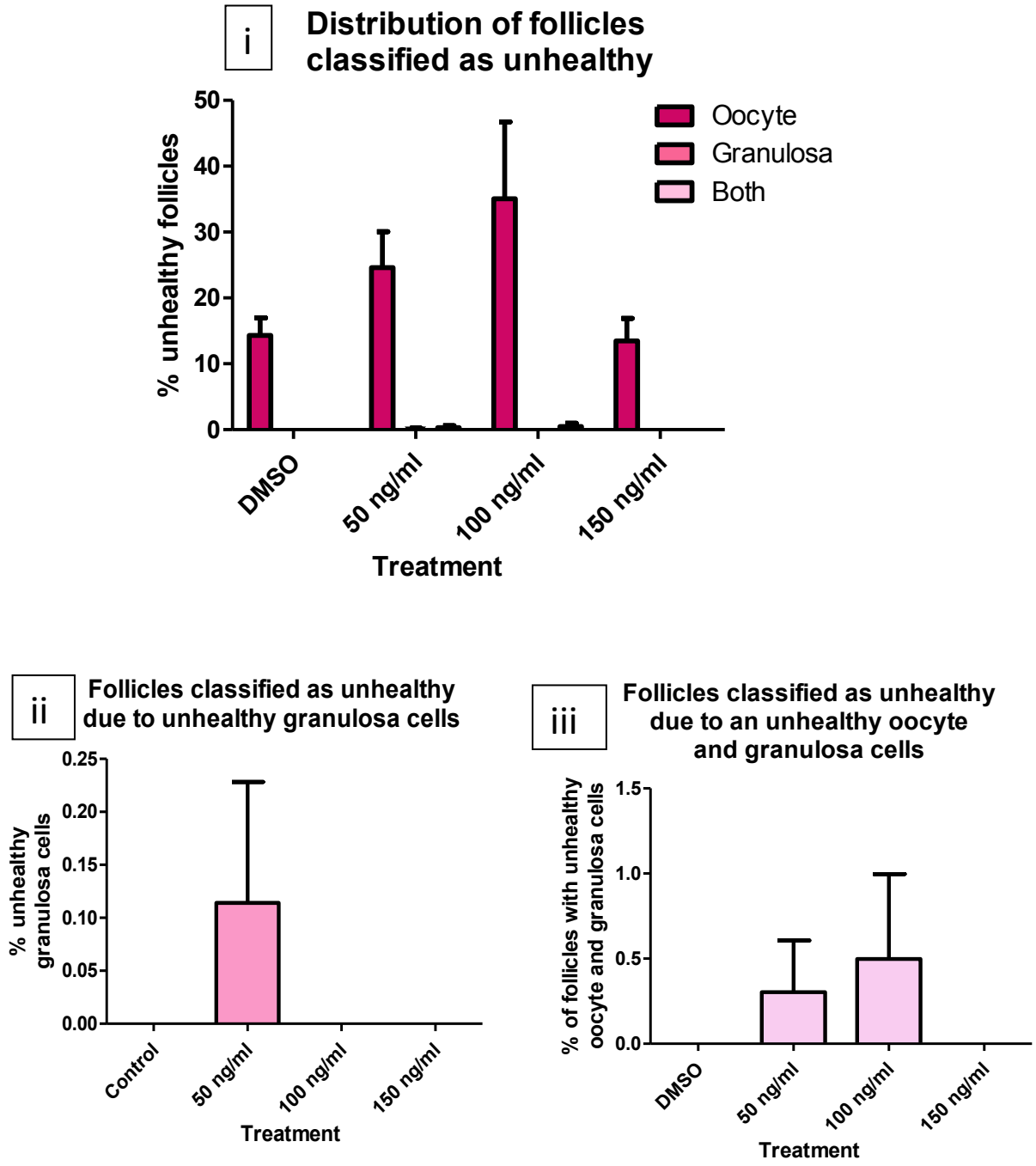


Figure 5.18. Distribution of follicles classified as unhealthy due to an unhealthy oocyte, granulosa cells or both. Unhealthy follicles were further classified into damaged cell types, with the vast majority of follicles being classified as morphologically unhealthy due to an unhealthy oocyte (i). Below 2% of follicles at the low dose were classified as unhealthy due to unhealthy granulosa cells (ii) and below 1% of follicles were classified as unhealthy due to both unhealthy oocytes and granulosa cells at the low and medium dose (iii). Bars denote mean + sem; n=8 for control and 10 µM, n=7 for 1 and 100 µM.

5.4.5. Expression of γH2AX, a marker for double strand DNA breaks in etoposide treated ovaries

Sections from cultured neonatal ovaries treated with 250 ng/ml etoposide and cultured embryonic ovaries that had been treated with 50, 100 or 150 ng/ml etoposide were stained for γH2AX (Figs. 5.19-20). There appeared to be an increase in cells from both the post-natal and pre-natal exposure groups expressing γH2AX. Following post-natal etoposide exposure, the expression was observed in both oocytes and a few somatic cells (Fig. 5.19), whereas within the embryonic ovaries that received the pre-natal exposure, γH2AX expression was observed primarily within the oocytes (Fig. 5.20).

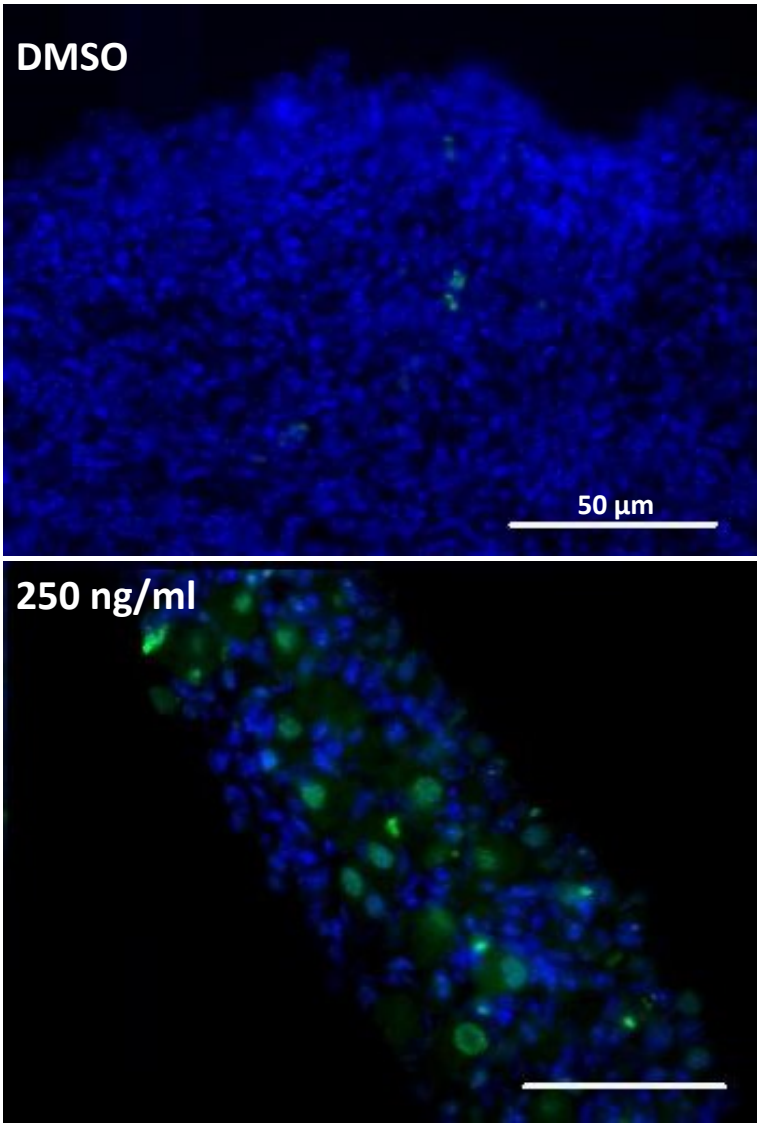
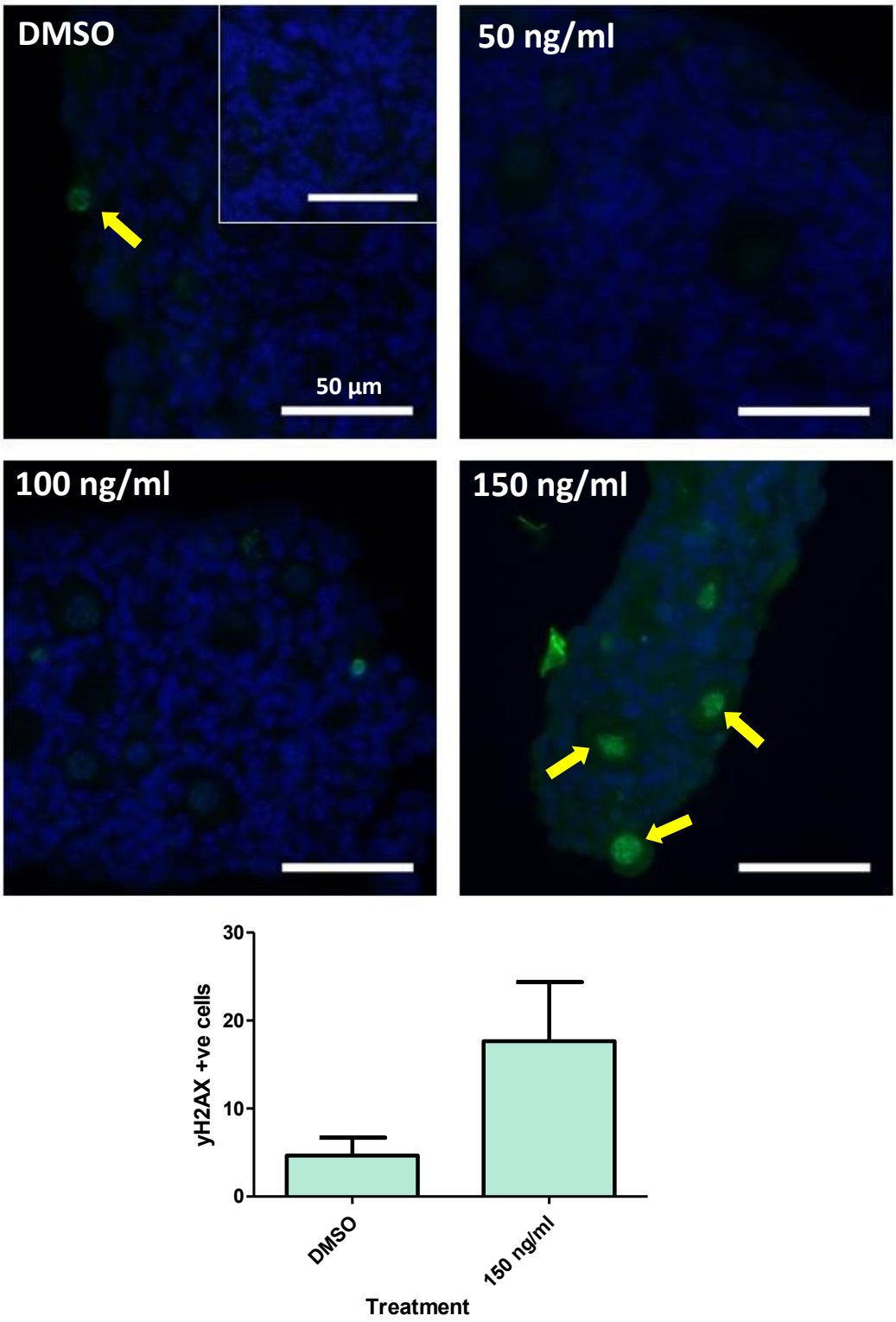


Figure 5.19. γH2AX expression in the cultured neonatal ovary following post-natal etoposide exposure. Neonatal ovaries cultured with DMSO or with 250 ng/ml etoposide for 6 days.The number of yH2AX positive cells in etoposide treated ovaries appears to increase. .



5.20. γH2AX expression in the cultured embryonic ovary following pre-natal etoposide exposure (i) and a histogram representing the number of γH2AX positive cells in DMSO vs. high dose ovaries (ii). E13.5 ovaries cultured for 6 days in the presence of DMSO or etoposide (50, 100 or 150 ng/ml), followed by another 6 days in culture media. Insert shows negative control. Yellow arrows depict examples of oocytes expressing γH2AX (i). There is a slight increase in the number of γH2AX expressing cells in etoposide treated ovaries, although this did not reach significance (ii) (n=3, p=0.136).

5.5 Discussion

5.5.1 The expression pattern of Topo IIα within the *in vivo* & *in vitro* mouse ovary.

Immunohistochemical detection of Topo IIα was carried out on cultured embryonic ovaries and *in vivo* ovaries. Ovaries were collected at a series of time-points from E13.5 through to end of culture (12 days), or up-to PND6 *in vivo*. In the pre-natal *in vivo* ovary, prior to follicle formation, Topo IIα was detected specifically within the germ cells. Around birth, when follicles began to form and flattened pre-granulosa became visible around the germ cells, the expression became localised to the surrounding granulosa cells and stromal cells. As follicles began to grow into transitional and primary follicles, expression became increasingly visible within the surrounding granulosa cells while still being expressed in the stroma. The same expression pattern was observed within the cultured oocytes, where the change in expression was observed between days 6 and 7 of culture, at the same time as follicles began to form within the culture system. This result is consistent with a previous observation in the *in vivo* rat ovary, where the expression of Topo IIα was observed within the germ cells during pre-natal ovary development, whereas it became localised to the surrounding somatic cells as follicles began to form a few days after birth (personal communication, Dr. N Powles-Glover, AstraZeneca) (Section 3.1.3). The change in localisation did occur a few days earlier in the mouse ovary, but this was expected as the rat ovary is slightly delayed when compared with the mouse ovary. Follicles form at birth in the mouse ovary, whereas they do not appear until a few days after birth in the neonatal rat ovary (Rajah et al., 1992, Sarraj and Drummond, 2012).

As far as I am aware, no prior studies had been carried out investigating expression pattern of Topo IIα within the rodent ovary. The expression pattern of Topo IIβ however had been previously described in the rodent ovary, where it was localised to the oocytes at all developmental stages. Topo IIβ was also weakly expressed in the granulosa cells of primordial follicles, becoming more pronounced in the granulosa cells of larger growing follicles (Zhang et al., 2013) (Section 1.4).

5.5.2. The effect of pre-natal AZTC exposure on ovarian follicles.

Ovaries exposed to the highest dose of AZTC (100 μM) had significantly fewer follicles remaining at the end of the culture when compared with control, low- and medium dose ovaries. When the follicles were classified into their follicle types, the number of

primordial follicles firstly appeared to increase slightly in a dose-dependent manner at the low (1 μM) and medium (10 μM) doses, although this was not statistically significant. At the highest dose however, the number of primordial follicles dropped significantly. There also appeared to be slightly fewer transitional follicles in the high dose ovaries although again, this was not statistically significant. AZTC therefore resulted in what appeared to be a slight rise in follicle numbers at the lower doses, but a near complete obliteration of follicles at the highest dose, with very few follicles remaining at the end of the culture period. When the percentage of follicle distribution was analysed, a similar effect was observed for the percentages of primordial follicles, with a slight increase at low and medium doses, but a significant drop at the highest dose. The percentage of transitional and primary follicles did however increase; the opposite effect to what was observed when total numbers were considered. This is probably better interpreted as result of the substantial loss of primordial follicles, leaving behind proportionally more transitional and primary follicles, rather than an induction in follicle growth by AZTC.

AZTC had an effect on follicle health within the cultured ovaries, where a slight increase in the number of unhealthy follicles was observed at the low dose, nearly doubling at medium doses, although this was not significant. At the highest dose however, this number dropped to below that of the control ovaries, being significantly lower than that of the medium dose ovaries. This result is most likely due to the large amount of follicles lost in the high dose ovaries, where the wave of apoptosis has already passed, with the few remaining follicles being healthy. This effect was further confirmed when the unhealthy follicles were classified into their follicle types and a dose-dependent significant increase in unhealthy follicles was observed at the low and medium doses, and again a significant drop in the number of unhealthy follicles at the highest dose when compared with the low and medium doses. The percentage of unhealthy follicle types showed no significant difference however, demonstrating that proportionally, there was no difference between the percentage of unhealthy follicles of treated and control follicles. It is difficult to draw conclusions from this result considering the extensive loss of follicles within the high dose ovaries. This was most likely due to a large increase in atretic follicles at an earlier time-point during the culture, which would have gone on to die. As a consequence, follicle numbers within the ovaries were reduced, leaving behind the few remaining healthy follicles.

5.5.3. Comparison of *in vivo* and *in vitro* AZTC studies

Many of the results observed here were consistent with the results from the *in vivo* study where female rats were dosed pre-natally and ovaries collected at PND15 (Chapter 3). Despite the obvious difference in species between the two studies (rat vs. mouse), the exposure windows were similar, in that exposure occurred during meiotic entry, germ cell nest formation and nest breakdown, meiotic arrest and potentially the earliest stages of follicle formation (*in vitro),* but not during the main bulk of follicle formation and initiation of growth. The exposure window *in vivo* does, however, start earlier than could be accomplished *in vitro,* or from the time of conception. This means that exposure *in vivo* also occurred during the time of germ cell migration and proliferation. Despite these differences in study design, the majority of the results between *in vitro* and *in vivo* did appear to complement each other, but only in regards to the low and medium doses of the *in vitro* study. Follicle numbers within exposed ovaries appeared to increase slightly at the low and medium dose *in vitro,* where follicle numbers were also significantly increased in the *in vivo* AZTC exposed PND15 rat ovaries. When the number of primordial follicles were examined within these ovaries, it became clear that the observed rise in total follicle numbers was due to an increase in primordial follicles, this effect reached significance *in vivo* but the same trend was also observed *in vitro.* Furthermore, the number of unhealthy primordial follicles significantly increased consistently both *in vivo* and *in vitro* at the low and medium doses. However, in cultured ovaries exposed to the high dose of AZTC, a large significant drop in the total number and primordial follicle numbers was observed, as well as a drop in the number of unhealthy follicles. This difference between *in vivo* and the high dose *in vitro* ovaries could be due to various reasons, making it difficult to effectively compare the two studies. Firstly it is not known how the *in vitro* doses compare with how much is reaching the ovaries *in vivo*. AZTC has to pass through the placenta before reaching the ovaries *in vivo*. This means that a lower concentration of AZTC might be reaching the *in vivo* ovaries as it might not pass as easily through the placenta, resulting in less exposure to the embryo. It is therefore possible that the doses reached *in vivo* are only equivalent to the low and medium doses *in vitro.* On the other hand, another possibility is that AZTC could accumulate in the placenta and in the embryo, with the embryo having a higher metabolic rate than the mother, this could, in fact, result in increased AZTC exposure of the embryo. Secondly, the cultured embryonic ovaries might be

more vulnerable and sensitive to the drug than those exposed *in vivo* and therefore a lower concentration *in vitro* could do more damage than the equivalent exposure *in vivo.* The cultured ovaries have been removed from their surrounding ovarian bursa and normal blood supply, meaning that AZTC might have more of a direct access to the follicles as the medium diffuses through the ovary, as opposed to being delivered through the blood supply *in vivo.* The culture environment will also most likely not provide the same support as the real life situation, meaning the ovarian cells might be more susceptible to damage or struggle to repair themselves. It is nevertheless possible that if the *in vivo* study had been carried at a higher dose then the same results might have been observed as in the *in vitro* study, where the primordial follicle numbers drastically dropped at the highest dose. Considering the effect of culturing the ovaries with 200 and 500 μM AZTC, where no follicles remain at the end of culture, it is highly likely that this would be the case. Unfortunately this could not be tested (Section 3.3.1). With regards as to whether the results observed following the *in vivo* PND 15 study (Section 3.4.2) were due to an observed litter-effect from only being able to use a litter born the one mother, or if it was a true finding, the findings presented here suggest that the previous *in vivo* results were a result of AZTC exposure to the pre-natal ovary due to the fairly close correlation between *in vivo* and *in vitro* studies. These results are encouraging as despite the difficulty in predicting the similarities between dosages, the results reflect each other closely when comparing the low and medium AZTC dosed *in vitro* ovaries to the *in vivo* exposed ones, suggesting that the embryonic ovary culture could potentially be a useful tool in future reprotoxicity studies as an investigatory tool for ovarian toxicants.

5.5.4 Effect of AZTC on meiosis

Embryonic ovaries that had been cultured in the presence of DMSO or the top dose of AZTC (100 μM) for 6 days were collected and meiotic spreads were carried out with immunofluorescent staining for Scp3 and Scp1. Unfortunately, due to the lower yield of oocytes from the AZTC treated ovaries, not enough nuclei were visible to carry out full analysis of the proportion of nuclei in leptotene, zygotene or pachytene. However, several nuclei were observed in which the homologous chromosomes appeared to have fully synapsed. Although it is possible that AZTC was interfering with meiosis, resulting in this reduced number of follicles surviving, some oocytes were able to progress to pachytene in the presence of AZTC. However, the loss of oocytes following

the high dose of AZTC could also be due to a different effect, such as on apoptosis for example, which might be un-related to meiosis. This study would therefore have to repeated to elucidate this further, with ovaries exposed to varying doses of AZTC. A higher yield of oocytes could then be quantified appropriately to determine if AZTC is, in fact, affecting meiosis.

5.5.5 Mechanism of action of AZTC

Since AZTC was added to the media only during the first 6 days of culture, exposure was limited to the time period that Topo IIα and Topo IIβ were expressed within the germ cells. Ovaries were placed in fresh control media at the 6th day of culture, and therefore the end of the exposure window coincided with the change in Topo IIα expression from germ cells to the surrounding somatic cells. The results described here coincided quite closely with the results outlined in Chapter 3 and therefore similar conclusions will be drawn about the effect of AZTC on the pre-natal ovary. AZTC was believed to potentially inhibit mammalian Topo II with the expected effect on proliferating cells and a reduction in oocyte number, although the start of the embryonic culture most likely does not overlap with the tail-end of proliferation, as it encompasses the beginning of meiosis. The role of Topo II in meiosis is still not fully understood, but the expression of both paralogues of Topo II was confined to the germ cells during this time. The slight dose-dependent increase in follicle numbers observed at the lower doses of AZTC was an un-expected result, yet consistent with previous observations *in vivo* (Section 3.4.2). A reduction in follicle number was observed during the embryonic ovary culture but only at the highest dose (100 μM). Hence, AZTC could potentially be inhibiting Topo II but could have an antagonistic effect at lower doses, only acting as a 'classic' Topo II inhibitor at the higher doses where we observe the consequent loss of follicles. There was no obvious effect on stromal cells which would have also been expected to become atretic following AZTC exposure as they are rapidly diving, although by the end of culture (6 days after the AZTC has been removed) these might have already died. No effect on granulosa cell health was observed, although this was expected. This is because AZTC was removed prior to the time that Topo II (α and β) was expressed within the granulosa cells and the phase of granulosa cell proliferation.

Topo II has previously been shown to be expressed in dividing cells during mitotic phases of the cell cycle although its presence within non-cycling cells is not clear (Heck

et al., 1988). Topo II has been localised at the bases of chromatin loops where it is closely associated with the stable maintenance of chromosomes (SMC) family of proteins and believed to regulate the topology of the loop. It was shown to have maximum condensation during mitosis and meiosis (Baguley and Ferguson, 1998). Here, the expression of Topo II was shown to be localised to germ cells during meiosis in the pre-natal ovary. Therefore, since AZTC exposure occurred during meiosis, at the point at which Topo II was expressed within the germ cells, a likely effect on meiosis was expected. Furthermore, the *in vivo* effect of AZTC on male rats elicited effects on spermatogenesis (Section 3.3.1). Unfortunately, the meiotic chromosome spreads did not yield enough nuclei to fully analyse meiotic stages effectively, although this was most likely due to the observed loss germ cells at this high dose. Despite this, several nuclei were observed where full synapsis had occurred. Furthermore, although fewer oocytes remained at the end of culture, the follicles that did survive were able to form follicles and from histological analysis the oocytes appeared to be at dictyate, (although a larger number of oocytes would have been required to investigate if there was a severe meiotic delay caused by AZTC). It is indeed possible that a few oocytes managed to develop to pachytene and that the majority were not able to enter leptotene, resulting in the severe lack of observed oocytes in prophase I, as the immunostaining only detects germ cells that have entered meiosis and are therefore at leptotene, zygotene or pachytene. This makes it difficult to comment on the effect of AZTC on meiosis at this point without further investigation. It is possible to speculate that AZTC was inhibiting Topo II, but perhaps Topo II is not crucial for prophase I of meiosis, or perhaps other enzymes might be present to compensate for the loss of Topo II activity. On the other hand, it is also possible that AZTC did not inhibit Topo II and was eliciting effects on the embryonic ovary through secondary or off-target effects.

A possible secondary target for AZTC is through inhibition of other enzymes that bear similar morphology to Topo II such as the 90 kDa heat shock protein family (Hsp90). Hsp90 are molecular chaperones ubiquitely expressed in eukaryotic cells. They play a fundamental role in the folding and activation of proteins that are essential for cell cycle regulation, steroid hormone signalling and response to cellular stress (Wandinger et al., 2008, Young et al., 2001). Hsp90 proteins are also key players in the maintenance of cellular homeostasis (Taipale et al., 2010). The structure of the N-domain of the Hsp90 molecule has similar topology to the N-terminal ATP binding domain of the Topo II

molecule (Dunbrack et al., 1997, Prodromou et al., 1997). Since the exact mechanism of AZTC inhibition on Topo II is unknown, the possibility that AZTC might be inhibiting other proteins with similar topology to Topo II cannot be excluded. If AZTC also acts by inhibiting Hsp90, this might contribute to the observed results in the ovary following AZTC exposure.

It must also be borne in mind that AZTC was developed as an antibacterial agent, but the rich ovary culture medium also contains another source of antibiotic in the medium, a penicillin-streptomycin (pen-strep) solution (Section 4.3.4). Although the pen-strep solution is not supposed to affect the ovaries within the culture system, there is a slight possibility that the pen-strep and AZTC might have additive effects which would not occur if AZTC was present within the culture media alone. However, this culture system is vulnerable to infections and could therefore not be carried out without including pen-strep added to the culture medium.

Differences have been shown between eukaryotic and prokaryotic Topo II (Gupta et al., 1987). In prokaryotes, the Topo II molecule consists of two sub-units: DNA gyrase A protein (GyrA) and DNA gyrase B protein (GyrB), making the active form a A_2B_2 heterodomier. In eukaryotes however, Topo II exists as a homodimer where the fused A and B sub-units are equivalent to the monomer (Kampranis et al., 1999). Consequently, certain Topo II targeted drugs have been reported to have different effects on prokaryotic vs. eukaryotic cells. Etoposide and Teniposide, for example, show high mutagenicity to mammalian cells at relatively low concentrations (50 ng/ml) but have very little effect on prokaryotic cells (Gupta et al., 1987). Since AZTC was being created as an antibacterial agent, targeting bacterial Topo II, it is therefore possible that AZTC might not elicit the same effects on Topo II in eukaryotic cells, despite homology between bacterial and mammalian Topo II.

5.5.6. The effect of pre-natal etoposide exposure on ovarian follicles.

Etoposide resulted in a dose dependent reduction on follicle numbers, reaching significance at the top dose of etoposide (150 ng/ml) when compared with controls and low dose ovaries. When follicles were distributed into their follicle types it became apparent that the reduction in follicle number was due to a reduction in primordial and transitional follicles, with a slight decrease in primary follicle numbers as well, although

this was not significant. The number of unhealthy follicles within etoposide treated ovaries showed a similar trend as those exposed to AZTC, with a slight, non-significant increase in the number of unhealthy follicles at the low dose, but dropping significantly at the high dose when compared with the low dose. This was observed for both for primordial and transitional follicles. When the proportion of unhealthy follicles was examined, there was a significant increase in the proportion of unhealthy follicles at the middle dose, but again, a drop in numbers at the highest dose. As already outlined above for AZTC, since the ovaries remain in culture for 6 days following the end of etoposide treatment, the most likely explanation for this is that the wave of apoptosis induced by the high dose of etoposide results at an earlier time-point in culture, causing the follicles to become atretic and die. As a consequence, fewer follicles remain at the end of culture, but the few remaining follicles are healthy. At lower doses, the follicles might not be as strongly affected, and follicles may therefore take longer to become atretic and die.

5.5.7. Etoposide resulted in increased DSB formation within exposed ovaries

Etoposide has been suggested to inhibit Topo II by interfering with the nick closure reaction, resulting in DNA fragmentation and increased DBSs within the DNA (Gupta et al., 1987). DSBs are highly deleterious lesions in genomic DNA that can be generated by Topo II poisons including etoposide. If DSBs are not efficiently repaired, chromosomal aberrations and apoptosis occurs. Here, immunofluorescent staining was carried out for the DSB marker, γH2AX, in neonatal ovaries cultured with etoposide and in embryonic ovaries that had been exposed to etoposide for the initial 6 days of culture, during meiosis, but had then been left in control medium for a further 6 days. DSBs play a crucial role in meiosis where they are used to mediate the immunoglobulin class-switch recombination event (Daboussi et al., 2002) and therefore γH2AX immunofluorescence staining could not be carried out on pre-natal ovaries, as DSBs normally occur during meiosis. A large increase in γH2AX staining became visible in ovaries exposed to etoposide both pre-natally (150 ng/ml) and post-natally (250 ng/ml), at doses that are considerably lower than the 5-60 μg/ml concentration measured in human plasma following treatment with etoposide (Hande *et al.,* 1984). This was an expected result as etoposide has previously been shown to induce DSBs, suggesting that etoposide utilises the mechanism of DNA strand breaks to induce cell death (Smart, 2008). γH2AX expression was primarily observed within the germ cells of pre-natally

exposed ovaries, whereas γH2AX expression was detected within the germ cells but also appeared to be expressed within the surrounding somatic cells in the post-natally exposed ovaries. This could potentially be due to etoposide exposure occurring during the phase of somatic cell proliferation in the post-natal ovary, affecting cell division, whereas in the pre-natal ovary culture, follicle formation occurs after etoposide has been removed from the culture medium and therefore granulosa cells were able to form and divide without any interference by etoposide. An interesting next step would be to confirm the observed differences in γH2AX expressions within germ and somatic cells between the pre- and post-natal exposure ovaries. This could be done by carrying out immunofluorescent staining for γH2AX in conjunction with cell specific markers for germ cells, such as vasa, MVH or GDF-9, for example.

5.5.8 How might etoposide affect the unborn ovary?

The results presented here show a concerning, dose-dependent effect of increased etoposide exposure that results in a reduction of the follicle pool and decreased follicle health, with a corresponding increase in DSBs. Although there are some differences between the mouse cultures described here and the real-life situation of *in-utero* human exposure, these results still indicate a cause for concern, in particular since etoposide has been prescribed to pregnant women. Although these women did give birth to healthy children, little can be done to investigate potential effects etoposide had on the developing ovary given the short follow-up time possible at present. The effects will not become apparent for another 6-30 years when the daughters born to these women begin their own menstrual cycles and/or try to conceive children of their own.

Inter-species differences are the primary concern at hand here, where effects observed in the mouse ovary might not be reflected in what is observed in the human ovary. It is possible that the cultured embryonic ovary might be more delicate, resulting in an increased effected at lower doses than what would be observed *in vivo.* Cultured ovaries might also have a higher rate of cellular uptake of etoposide than what might occur *in vivo.* Furthermore, *in-utero,* some of the etoposide might be filtered through the placenta, although the majority of the current evidence points towards the ability of etoposide to cross the placenta and reach the developing fetus (Ostrea et al., 2004, Hengstler et al., 2002). Despite this, the exposed cultured ovaries demonstrate clear dose-dependent effects following etoposide exposure which should be carefully

considered, as it is likely that if etoposide targets the DNA within mouse germ cells in culture, that similar effects might be observed in the human fetus *in vivo*. Moreover, topoisomerase-targeted drugs can elicit genetic mutations which could be transmitted to future generations and therefore the effects of the pre-natal etoposide exposure would perhaps not become clear until after two generations.

The exposure of cultured mouse ovaries to another chemotherapy drug, cisplatin, at the same concentrations as are measured in patient serum levels, has been shown to induce in ovary damage. Cisplatin is known to induce moderate damage to the patient's fertility, demonstrating that the effects observed using mouse ovary culture techniques can correlate closely with the reproductive effects that occur following chemotherapy treatment in humans (Morgan et al., 2013).

5.5.9 Comparing the effects of pre-natal AZTC and etoposide exposure

As outlined above, both AZTC and etoposide resulted in an unusual dose-response curve in that a higher proportion of unhealthy follicles were observed at lower and medium doses than at the top doses. The results did not, however, complement each other with regards to follicle numbers within the ovaries. AZTC initially resulted in an increase in follicle numbers followed by a drop at the top dose, whereas follicle numbers reduced in a dose-dependent manner following etoposide exposure. It is worth noting that the observed increase in follicle numbers following AZTC exposure is a fairly unusual result from prior work in the Spears Laboratory with various reproductive toxicants and chemotherapy drugs (Personal Communication, Prof. N. Spears), as the more commonly observed effect of drug-induced ovary disruption, is a loss in follicle numbers. One potential explanation for this result is that AZTC is, in some way, affecting the wave of apoptosis associated with germ cell nest breakdown which is believed to remove any germ cells that are of a lesser quality (Pepling and Spradling, 2001). AZTC might inhibit apoptosis, or it might disrupt the process of germ cell nest breakdown and result in more germ cells forming. Indeed, some multioocyte follicles have been observed, but this has also been observed in control ovaries and is therefore not necessarily an effect of AZTC. Whether AZTC inhibits mammalian Topo II in a manner similar to etoposide is difficult to comment on and would require further studies, as the results here do not correspond as closely as was expected. AZTC could still be inhibiting mammalian Topo II but potentially via an alternate pathway.

5.5.10. Could the embryonic ovary culture be a potential tool for use in future reproductive toxicity studies?

The embryonic ovary culture used here to test the potential reproductive effects of two Topo II inhibitors: AZTC and etoposide. The *in vitro* effects of AZTC were compared with effects from the *in vivo* study where the window of exposure resembled that of the embryonic ovary culture. The results from the PND15 *in vivo* AZTC study and the embryonic ovary culture correlated quite closely, but only in regard to the low and medium dosed cultured ovaries. Despite this, it is a very encouraging result as it suggests that the culture system might be a good and potentially, a more sensitive tool for preliminary investigations into potential reproductive effects of novel pharmaceutical compounds.

The second drug tested here, etoposide, showed surprising results, where at doses considerably lower than the serum level following etoposide treatment, major effects were observed on the follicle pool and ovary health. This is a worrying result as, if it translates to the human, it could suggest that girls born to mothers that have had to undergo chemotherapy might suffer from fertility problems later in life. These results do correlate with previous studies where ovarian effects of etoposide were observed in mice and hamsters, with increased frequency of aneuploid oocytes and chromosome aberrations (Mailhes and Marchetti, 1994, Tateno and Kamiguchi, 2001a).

It should be borne in mind that chemotherapy treatment does not usually just involve one chemotherapy drug, which could further cause further complications when trying to elicit which drug has the reproductive effect, or whether a combination of drugs aggravates the reprotoxicity effects. Therefore, *in vitro* cultures where drugs can be used alone or in combination could be an important and effective tool in determining the mechanisms of action of drugs, and how effects may differ when used alone or in combination as is frequently observed with chemotherapy drugs.

5.6 Conclusion

Embryonic ovaries cultured with the low and medium dose of AZTC demonstrated effects that were consistently similar to what was observed in the PND 15 *in vivo* ovary following AZTC exposure. Another Topo II inhibitor and potential ovarian toxicant was then used to test the efficiency of the embryonic ovary culture system. Etoposide elicited effects on the follicle pool and health, suggesting potential effects on fertility on girls born to mothers who have had to undergo etoposide treatment during pregnancy. These results suggest that the embryonic ovary culture could be used as a preliminary screening tool for novel or pre-existing pharmaceutical drugs or potential reproductive toxicants. Furthermore, these cultures could be used as a way to elucidate if chromosomal sensitivity of oocytes to certain compounds or drugs is dependent on meiotic stages.

5.7 Future directions

In the future, it would be interesting to carry out further embryonic ovary cultures using other suspected or known reproductive toxicants, or Topo II inhibitors. These could include drugs such as teniposide and doxorubicin (Hande, 1998), or chemicals found in cigarette smoke, such as DMBA or B[a]P, that have been shown to affect the pre-natal ovary *in vivo* (Borman et al., 2000, Igawa et al., 2009, Matikainen et al., 2002, Mattison and Thorgeirsson, 1979). Future work could also involve repeating the meiotic chromosome spreads on more AZTC-treated ovaries to obtain a larger number of oocytes so that the proportion of oocytes in the different stages of early prophase-I could be analysed effectively. It would also be interesting to carry out meiotic chromosome spreads on etoposide treated ovaries. The γH2AX immuno could also be carried out on AZTC treated ovaries, to investigate if AZTC elicits the same DSBs in culture as is observed following etoposide treatment. Finally, it would be interesting to investigate if a single high dose of AZTC or Etoposide elicited a different response than the continuous dose, as was carried out here, since cells can develop a resistance to drugs. A single high dose exposure might therefore produce a more devastating effect than continued exposure of etoposide at a low or medium dose.

Chapter 6
Effects of AZTC on the post-natal ovary in vitro

6.1. Introduction

Topoisomerases, AZTC and etoposide have already been described in detail in previous chapters (Sections 1.4 and 5.1.1, respectively) and will therefore not be outlined again here.

6.1.1 The rodent neonatal ovary culture.

In rodent ovaries, primordial follicle assembly occurs around the time of birth. The process of follicle formation and the subsequent initiation of follicle growth are two separate processes both covered by the culture of neonatal ovaries (Devine *et al.* 2002b). The neonatal ovary culture involves culturing of a whole rodent ovary from after birth for up to 20 days (Eppig and O'Brien, 1996, Parrott and Skinner, 1999, O'Brien et al., 2003, Devine et al., 2002b). It is an appropriate culture system for studying the biology of primordial follicle assembly and the primordial-to-primary follicle transition, and is thus a valuable asset to toxicological research to identify potentially hazardous compounds that could interfere with these processes, or for scientists looking to investigate factors involved in early follicular growth.

6.1.2. Strain differences in ovarian follicles in mice

The mouse is a commonly used model in mammalian research studies, including studies in reproduction and reproductive toxicology. Since both women and mice share crucial reproductive aspects such as being born with a finite number of follicles, the mouse is a good model system for studying effects of potential ovotoxicants on the ovary and follicle pool.

There are various mouse strains available to use in laboratory studies, including CD-1, C57BL/6J, C57BL/10, CBA, BALB/c, C3H, FVB, and SWR. These strains are widely used for transgenic and gene knockout technologies as well as in reproductive toxicity studies. Inter-strain differences have been reported in various strains of mice, including susceptibility to fibrosis, drug metabolism, sensitivity to hormones, testosterone levels, ovary function, ovotoxicity and follicle endowment (Mattison and Thorgeirsson, 1979, Canning et al., 2003, Thomas et al., 2010, Pepling et al., 2010, Brouillette et al., 2004, Walkin et al., 2013). Some of these differences can be explained through years of inbreeding, where some animals (such as CD-1 mice) were selected due to their larger litter sizes, which is a result of an increased ovulation rate. Over the years, this may

have altered the follicle population and ovarian responsiveness to gonadotropins, including a decrease in negative feedback on gonadotropins. As a result, CD-1 mice are more resistant to the effects of estrogen exposure, including the effects of certain estrogenic endocrine disruptors (Spearow et al., 1999). It is therefore important to be aware of inter-strain differences when it comes to investigating potential ovotoxicants, as choosing one strain over another may result in diverse outcomes, where CD-1 mice might not respond to a xenobiotic in the same manner as other mouse strains, resulting in a potential endocrine disruptor 'slipping under the radar'. Despite this, relatively little research has gone into investigating the differences between different strains of mice, in particular regarding the reproductive axis. This emphasizes the need to identify and study genetic variation between mouse strains, to further understand their sensitivity to gonadotropins, differences in ovarian function, vulnerability to disease and metabolic rate, to name a few.

6.2. Aims

The aim of this work was to carry out neonatal ovary cultures to investigate the effects of AZTC on the post-natal ovary, to compare with the effects with those observed when AZTC exposure occurs pre-natally. Another Topo II inhibitor, etoposide was then selected as a second study compound to evaluate its effects on the post-natal ovary compared with the effects of AZTC.

6.3 Materials and Methods

6.3.1. Animals

An initial experiment was set up where newborn (P0) female offspring from C57BL6 x CBA crosses (F1) were collected and ovaries were dissected for a 6 day culture. However, due to low litter numbers in the C57BL6/CBA mice this study was repeated with CD-1 mice, because the CD-1 pairs were having considerably larger and more frequent litters than the C57BL6/CBA pairs. Furthermore, CD-1 mice had already been used for the embryonic ovary cultures due to their large litter sizes (Chapters 4 and 5). CD-1 females were therefore selected for all subsequent newborn ovary cultures to ensure consistency between pre- and post-natal ovary cultures and allow direct comparisons.

6.3.2. Neonatal ovary culture

Ovaries from P0 or P4 CD-1 or F1 female pups were dissected and placed in pre-warmed L-15 medium as previously described (Section 2.1). Neonatal ovaries (P0 or P4) were cultured for 6 days on a floating membrane on simple medium in 24-well plates (Sections 2.1.2-2.1.3). The medium was placed in the incubator for 30 minutes to equilibrate before ovaries were placed on the membranes.

6.3.3. Experiment 1: Assessment of Topo IIα expression in the cultured neonatal mouse ovary.

Newborn (P0) ovaries (CD-1) were cultured for 6 days as outlined above, and were collected at each day of culture, on days 1-6. Ovaries were washed in 1xPBS for 5 minutes and fixed in 70% buffered formalin and processed as outlined previously (Section 2.2). *In vivo* mouse ovaries had already been collected from P0-P6 (Section 5.3.3). The expression pattern of Topo IIα in cultured neonatal ovaries was compared with ovaries at the equivalent stage *in vivo.*

6.3.3.1 Immunohistochemistry for Topo IIα

Topo IIα expression was detected in the ovary following the same protocol as outlined previously (Section 5.3.3.1). The primary antibody was a rabbit polyclonal anti-Topoisomerase IIα (Abcam, ab52934) and the secondary antibody was a biotinylated goat-anti rabbit (Dako); both were diluted at a 1:200 dilution in NGS/BSA/PBS (Section 2.4). Slides were blocked in the NGS/BSA/PBS solution before applying the primary antibody. The primary antibody was left on the slides overnight at 4˚C (Section 2.4.4). Slides were incubated with the secondary antibody for one hour at room temperature (Section 2.4.5). Topo IIα was detected with DAB (Section 2.4.6).

6.3.4. Experiment 2: P0 and P4 ovary cultured with AZTC

An initial dose-response study was carried where the medium was supplemented with AZTC at final concentrations of 1, 10, 100 and 500 μM AZTC in DMSO. The final concentrations of 1, 10 and 100 μM were selected for the study due to poor follicle morphology of ovaries cultured at higher doses. P0 (experiment 2a) or P4 (experiment 2b) ovaries were cultured for 6 days with either DMSO, 1, 10 or 100 μM AZTC. For the P0 experiment, a total of 5 ovaries were assessed for each treatment group from 3 independent cultures. For the P4 experiment, a total of 4 ovaries were assessed for each treatment group, from 2 independent cultures.

6.3.5. Experiment 3: PND 0 ovaries cultured with etoposide.

The effect of another Topo II inhibitor, etoposide, was also investigated on the neonatal ovary, to investigate if the effects of AZTC exposure correlated with that of a known Topo II inhibitor.

Initially, a dose response study was carried out on cultured P0 CD-1 mouse ovaries, where ovaries were cultured for 6 days with either DMSO, or etoposide at final concentrations of 25 ng, 250 µg or 25 mg/ml in DMSO throughout the culture period. This range of doses was selected to cover the plasma concentrations (5-60 µg/ml) reached in patients following etoposide treatment (Section 5.3.5). A total of 6 ovaries were assessed for each treatment group, from 4 independent cultures.

Final etoposide concentrations of 50, 100 and 150 ng/ml were selected for experiments. Ovaries were cultured for 6 days as outlined previously (Section 2.1).

6.3.6. Histological examination of cultured ovaries

At the end of culture, ovaries were washed in 1xPBS and placed in either Bouins fixative for 1-2 hours or in 10% buffered formalin overnight (Section 2.2.1). Ovaries were washed in 70% alcohol and left for 2-24 hours in 70% ethanol with eosin. Ovaries were then placed in tissue processing cassettes and processed (Section 2.2.2) before being embedded in plastic moulds and filled with wax. 5 µm sections were cut and every 6th section was H&E stained (Section 2.2.4) and analysed for follicle numbers, types and health. Follicles were counted blind to treatment and categorised as outlined previously (Section 2.3).

There were several ways in which the data could be analysed and presented, where either total follicle number (healthy, unhealthy, types, etc), or the percentage (proportion) of follicles were presented. While neither method is necessarily superior and examining both gives a better idea, or the 'bigger picture' of what is happening within the ovary, up-on reflection it was decided to present the data as percentages of total follicle numbers. This was the chosen method as I felt this demonstrated better the true distribution of follicles (healthy and unhealthy), especially in ovaries where total follicle numbers had decreased. The data was also analysed in terms of total numbers but these results were not included in this chapter to avoid complications when it came

to comparing all the different analyses (total numbers, percentage, follicle distributions, health, etc.) as well as comparing P0 vs P4 ovaries, AZTC and etoposide, and F1 vs CD-1. The excluded tables have therefore been placed in the Appendix instead (See Appendices C-F).

6.3.7. Statistical Analysis

Graphpad Prism was used for all statistical analyses of follicle number and distribution between DMSO control ovaries and ovaries exposed to AZTC or Etoposide as outlined previously (Section 3.3.3).

6.4 Results

6.4.1. Experiment 1: Expression pattern of Topo IIα in the cultured neonatal ovary

After one day of culture, Topo IIα expression could still be observed within many germ cells, as well as some stromal cells. By the second day of culture, very few germ cells and stromal cells were expressing Topo IIα. Topo IIα became limited to granulosa cells around days 3-4 of culture, with germ cells no longer expressing Topo IIα. By the fifth and sixth day of culture, Topo IIα expression was confined to the granulosa cells of growing follicles, although not all granulosa cells were found to be expressing Topo IIα. Some expression was also observed in stromal cells at this point (Fig. 6.1). The expression pattern correlated fairly closely with the *in vivo* ovary (Fig. 5.3, repeated in Fig. 6.2).

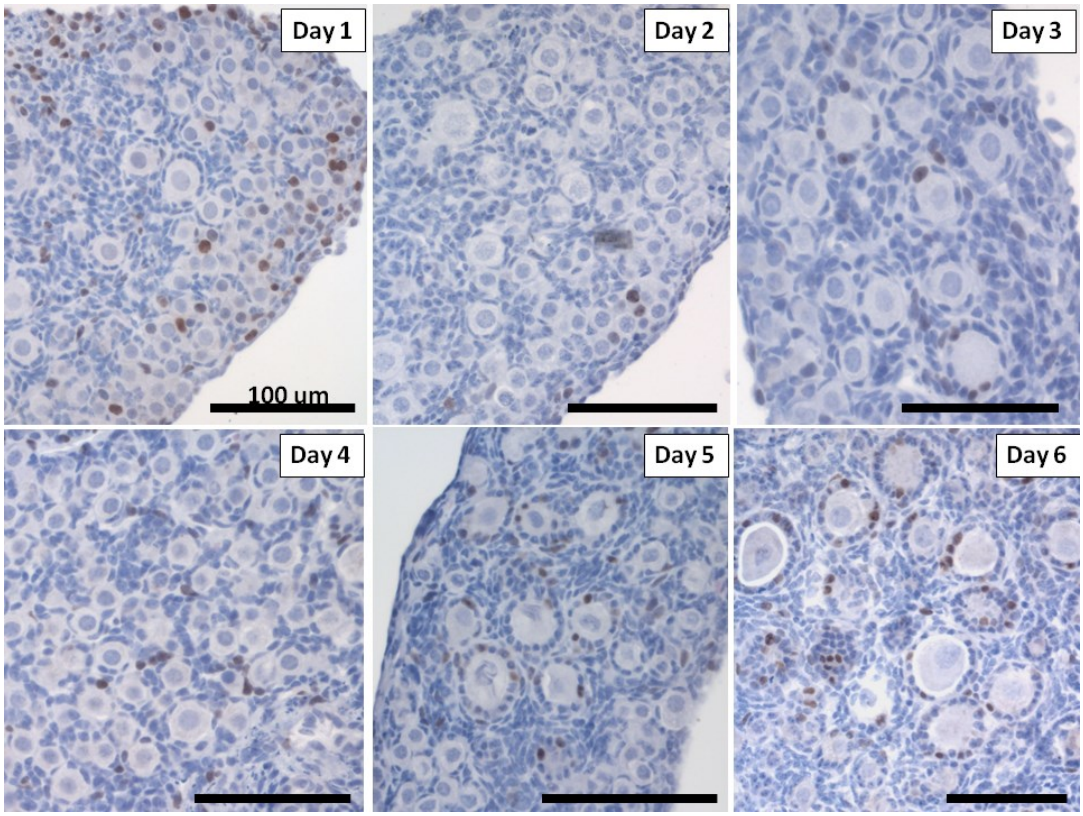


Figure 6.1. Expression pattern of Topo IIα in the cultured neonatal mouse ovary. Topo IIα was still detected within germ cells during the first two days of culture, but became localised to the surrounding granulosa cells and stromal cells around the third day of culture. By the end of culture, Topo IIα was expressed mainly in the granulosa cells of growing follicles and in some stromal cells.

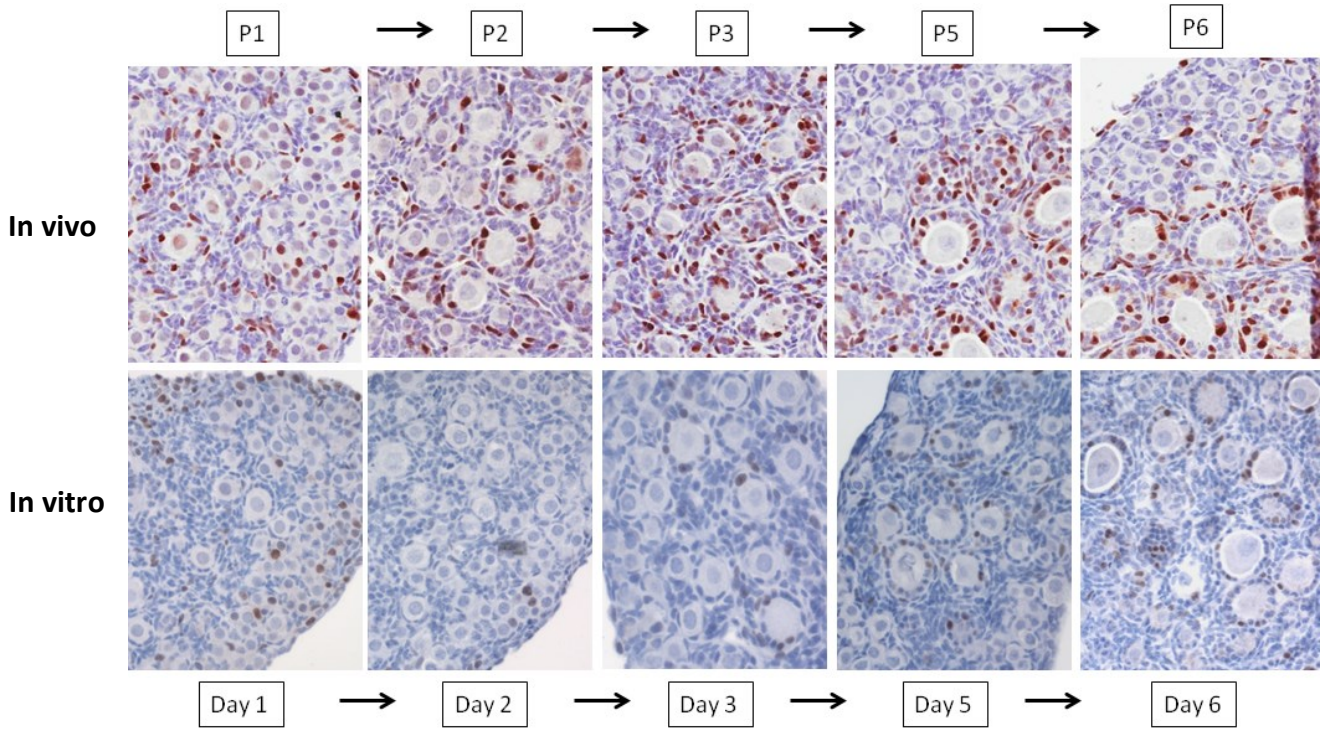


Figure 6.2. Comparison of the expression pattern of Topo IIα in the *in vivo* mouse ovary vs. the cultured neonatal mouse ovary. The same expression pattern was observed in the neonatal cultured ovary as was previously seen in the *in vivo* ovary. The top line of images here is repeated from Fig. 5.3. Topo IIα expressed was still observed within some germ cells around and shortly after birth, and in the first two days of culture. Expression of Topo IIα was then found exclusively within the somatic cells, in the granulosa and stromal cells around day 2-3 *in vivo* and around the 3rd day of culture.

6.4.2. Experiment 2: Effects of AZTC on the cultured newborn mouse ovary

6.4.2.1. Dose response study

Following the initial dose response study, no obvious histological effects were observed at the 1, 10 and 100 µM concentrations following a 6 day culture of newborn mouse ovaries (P0) with AZTC. At the 500 µM concentration however, the ovaries were shrunken and the ovarian stroma appeared to be very morphologically unhealthy and atretic, with no remaining follicles (Fig. 6.3).

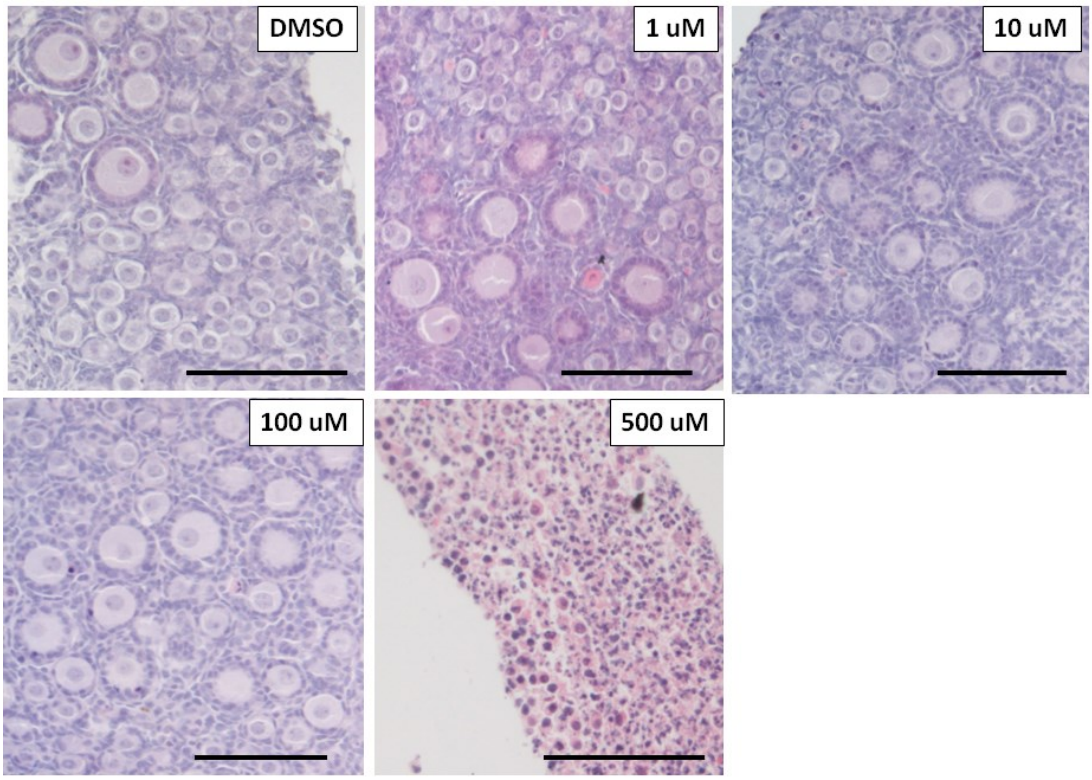


Figure 6.3. Dose response study with AZTC. Photomicrographs of ovary sections that had been treated with DMSO or AZTC at final concentrations of 1, 10, 100, or 500 µM. Ovaries exposed to 500 µM appeared very atretic with poor ovary morphology and no remaining follicles. Ovaries at lower concentrations contained healthy follicles at stages ranging from primordial to primary, with some secondary follicles visible as well.

6.4.2.2. Experiment 2a: Newborn ovary cultures with AZTC (CD1)

Newborn (P0) CD1 ovaries were cultured with DMSO or 1, 10 or 100 μM AZTC for 6 days (n=6 for all treatment groups) as described previously (Section 2.1). At the end of the 6 day culture (n=5 for all groups), there was no significant effect on the total number of follicles within the ovaries. There appeared to be a slight, dose-dependent reduction in follicle number with increasing AZTC dose, but this was not statistically significant (p=0.287) (Fig. 6.4i). AZTC had no significant effect on the percentage of primordial (p=0.424), transitional (p=0.474) or primary (p=0.213) follicles. There was, however, a significant decrease in the percentage of secondary follicles with increasing AZTC-dose (0.029) with the two-way ANOVA, but this was not supported by post-hoc tests (Fig. 6.4ii).

AZTC did not have an effect on the percentage (p=0.098) of unhealthy follicles within AZTC-treated ovaries, where the trend observed did not follow a regular dose-response pattern (Fig. 6.5i). That is, a slight increase in the proportion of unhealthy follicles was observed up-to the middle dose, which dropped again at the top dose. When the distribution of unhealthy follicles was examined, a significant increase was observed in the percentage of unhealthy transitional follicles at the medium dose when compared with controls (p=0.033) (Fig. 6.5ii), with this number dropping again at the top dose. A similar trend was observed for the percentage of unhealthy primordial follicles as well, but this did not reach statistical significance (p=0.387) (Fig 6.5ii).

In order to determine the cell type responsible for the follicles being classified as unhealthy, and to determine which cell type was being targeted by AZTC, follicles were further classified into unhealthy due to the oocyte only, granulosa cells only, or both due to an unhealthy oocyte and granulosa cells. Nearly all the unhealthy follicles within the AZTC treated CD-1 P0 ovaries were classified as such due to an unhealthy oocyte (Fig. 6.6). There was a slight increase in the proportion of follicles classified as unhealthy due to an unhealthy oocyte, up-to the medium dose, but this did not reach significance (p=0.089) (Fig 6.6i). A very small proportion (less than 1%) of follicles had been classified as unhealthy due to unhealthy granulosa cells.

There appeared to be slightly more unhealthy follicles due to unhealthy granulosa cells in control ovaries when compared with treated ovaries but this was not significant ($p=0.185$) (Fig. 6.6ii).

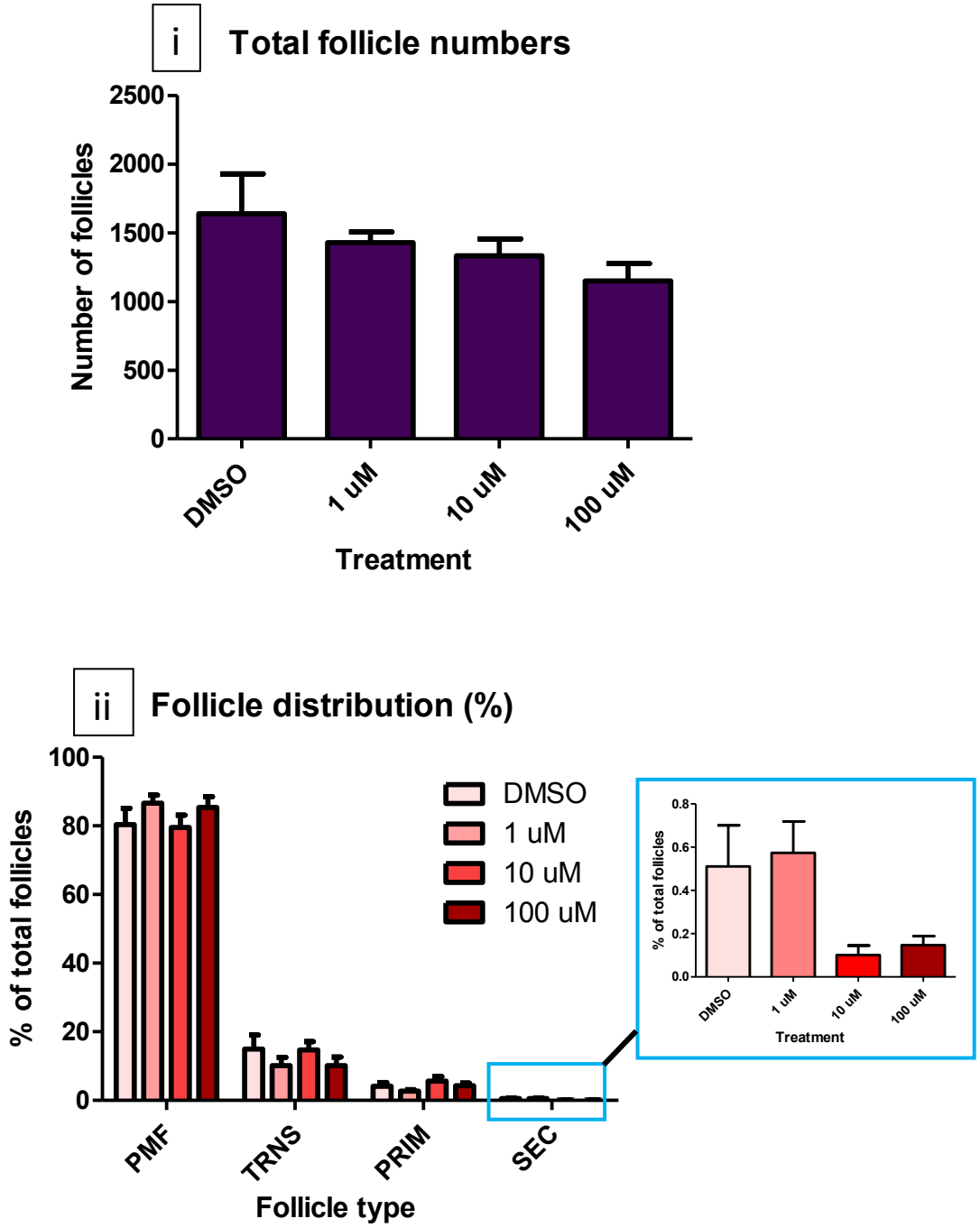


Figure 6.4. Total number of follicles within CD-1 P0 cultured ovaries. AZTC had no significant effect on the total follicle number within exposed ovaries compared with controls (p=0.287). A non-significant, dose-dependent trend was however observed where exposed ovaries appeared to have slightly fewer follicles with increasing dose of AZTC (i). AZTC had no significant effect on the distribution of follicles within the neonatal ovary when the percentage of total follicles was examined (ii). Bars denote mean + sem; n=5 for all groups. PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary.

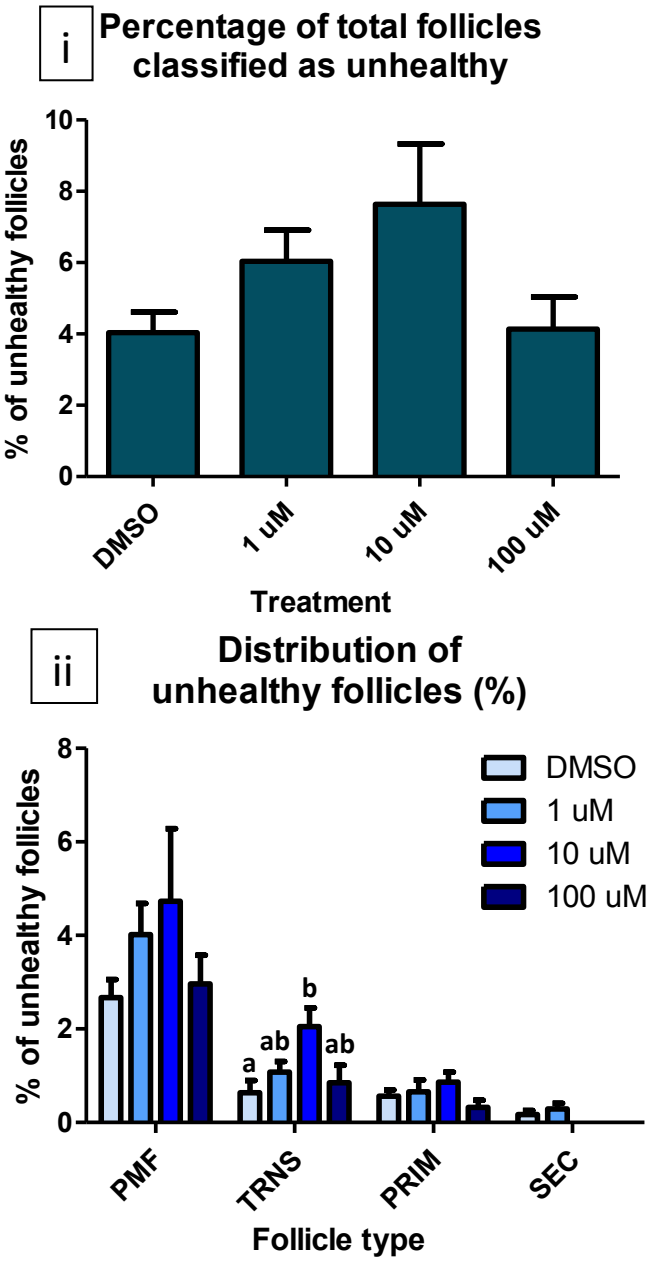


Figure 6.5. Follicle health within CD-1 P0 AZTC exposed ovaries. A slight non-significant increase was observed in the percentage of unhealthy follicles at the low and medium doses of AZTC, but this number dropped at the high dose (p=0.240) (i). Upon analysing the distribution of unhealthy follicles, the same trends were observed for the percentage of unhealthy follicles, where, again, a slight non-significant increase of unhealthy primordial follicles at the low and medium doses but a drop at the highest dose (p=0.387). The percentage of transitional follicles did significantly increase at the medium dose (p=0.033) and dropping at the highest dose (ii). Bars denote mean + sem; n=5 for all groups PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different (p<0.05).

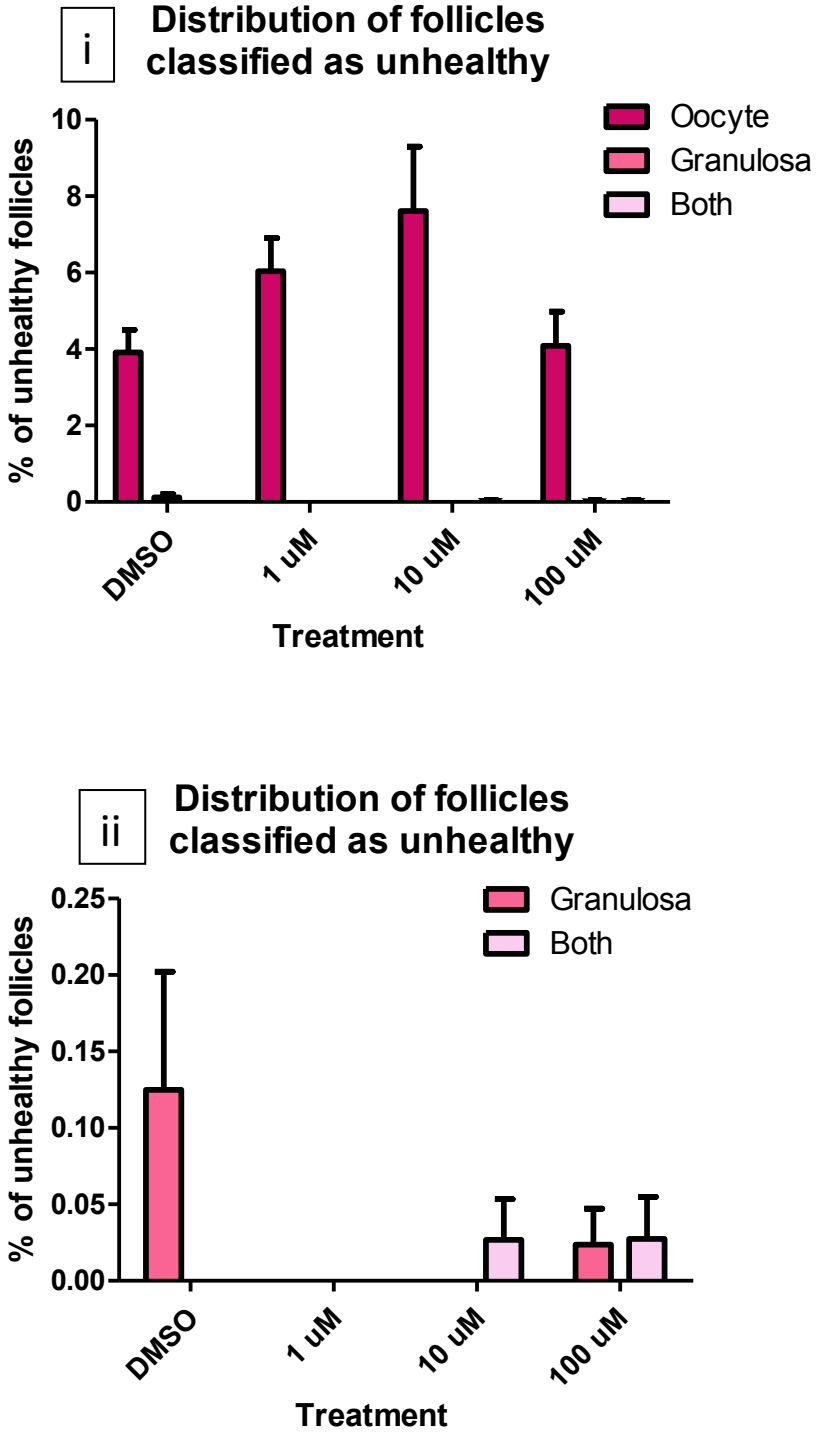


Figure 6.6. Distribution of follicles classified as unhealthy due to an unhealthy oocyte, granulosa cells or both. Unhealthy follicles were further classified into damaged cell types, with the vast majority of follicles being classified as morphologically unhealthy due to an unhealthy oocyte (i). There were no significant effects on the percentage of unhealthy follicles due to unhealthy granulosa cells or due to both unhealthy oocytes and granulosa cells ($p=0.1853$) (ii). Bars denote mean + sem; n=5 for all groups.

6.4.2.3. Experiment 2b: Neonatal (P4) ovary cultures with AZTC

Neonatal (P4) CD-1 mouse ovaries were collected and cultured for 6 days with DMSO or with AZTC at final concentrations of 1, 10 or 100 μM, as outlined previously (Section 2.1). Since Topo IIα was still expressed within the germ cells up-to 2 days after birth, this experiment was carried out to investigate whether a different effect might be observed if AZTC exposure occurred when Topo IIα was expressed within somatic cells of the ovary.

AZTC resulted in a slight, but non-significant reduction in total follicle number in all ovaries exposed to AZTC (p=0.075) (Fig 6.7i). When the follicles were distributed into their respective types, however, there was a significant reduction in the proportion of primordial follicles, but only at the medium dose (p=0.028). There was also a significant increase in the proportion of transitional follicles in the medium dose ovaries compared with controls (p=0.031) (Fig. 6.7ii).

When follicle health was examined, there was a significant increase in the proportion of unhealthy follicles at the medium dose (p=0.004) (Fig. 6.8i). The high dose ovaries did have a slightly higher proportion of unhealthy follicles when compared with controls, but this did not reach statistical significance (Fig. 6.8i). When examining the distribution of unhealthy follicles, a significant increase in the percentage of unhealthy primordial (p=0.010) and transitional (p=0.002) follicles was observed at the medium dose when compared with control and low dose ovaries (Fig. 6.8ii), followed by a reduction in percentage of unhealthy primordial follicles at the high dose, although the number was still relatively higher than controls, but not significantly so.

In order to determine the cell type responsible for the follicles being classified as unhealthy, and to determine which cell type was being targeted by AZTC, follicles were further classified into unhealthy due to the oocyte only, granulosa cells only, or both. Nearly all the unhealthy follicles within the AZTC treated embryonic ovaries were classified as such due to an unhealthy oocyte. The proportion of follicles

classified as unhealthy due to an unhealthy oocyte significantly increased, similarly as in Fig. 6.8, at the medium dose ($p=0.006$) (Fig. 6.9).

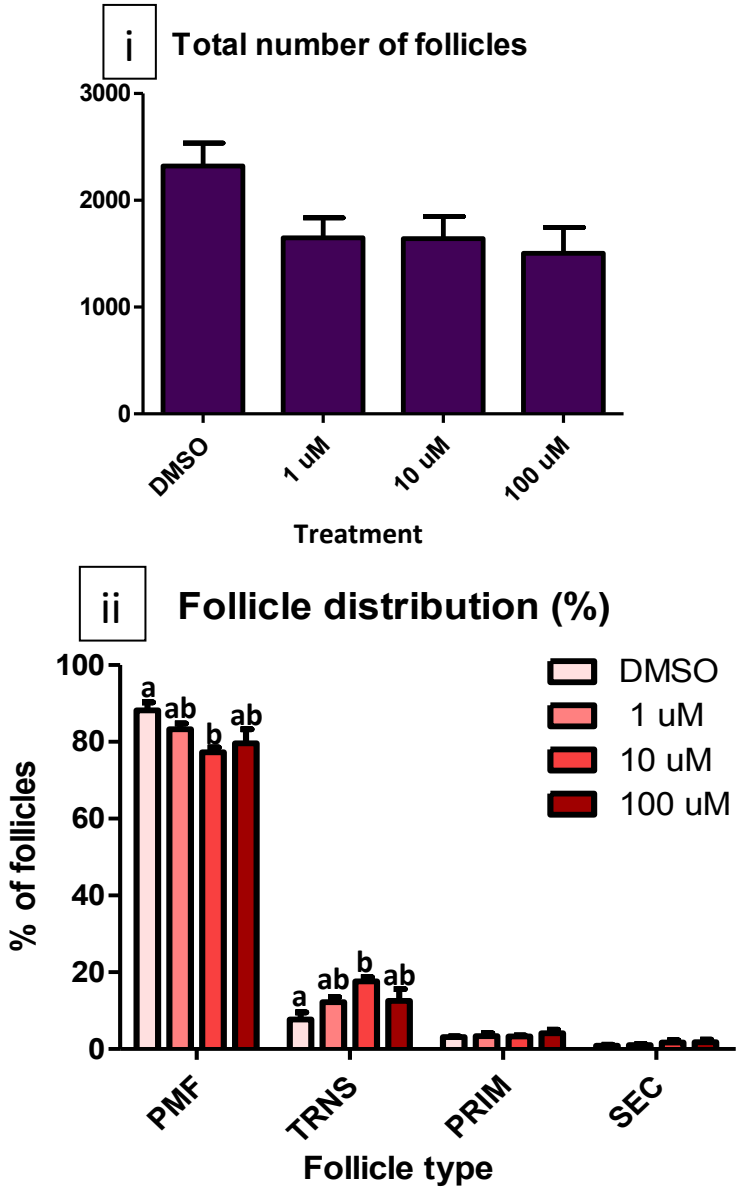


Figure 6.7. Total number (i) and distribution (ii) of follicles within CD-1 P4 ovaries cultured with AZTC. AZTC had no significant effect on follicle numbers within exposed ovaries (p=0.075), although all ovaries receiving AZTC treatment consistently contained slightly fewer follicles than controls (i). AZTC resulted in a significant reduction in the percentage of primordial follicles (p=0.028) in medium dose ovaries when compared with controls, and similarly, a significant increase in the percentage of transitional follicles was observed at the medium dose when compared with controls (p=0.031). No significant effect was found on the percentage of primordial or transitional follicles within low or high dose ovaries, or on other follicle types within treated ovaries (PRIM, p=0.644 . SEC, p=0.447) (ii). Bars denote mean + sem; n=4 for all groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different (p<0.05).

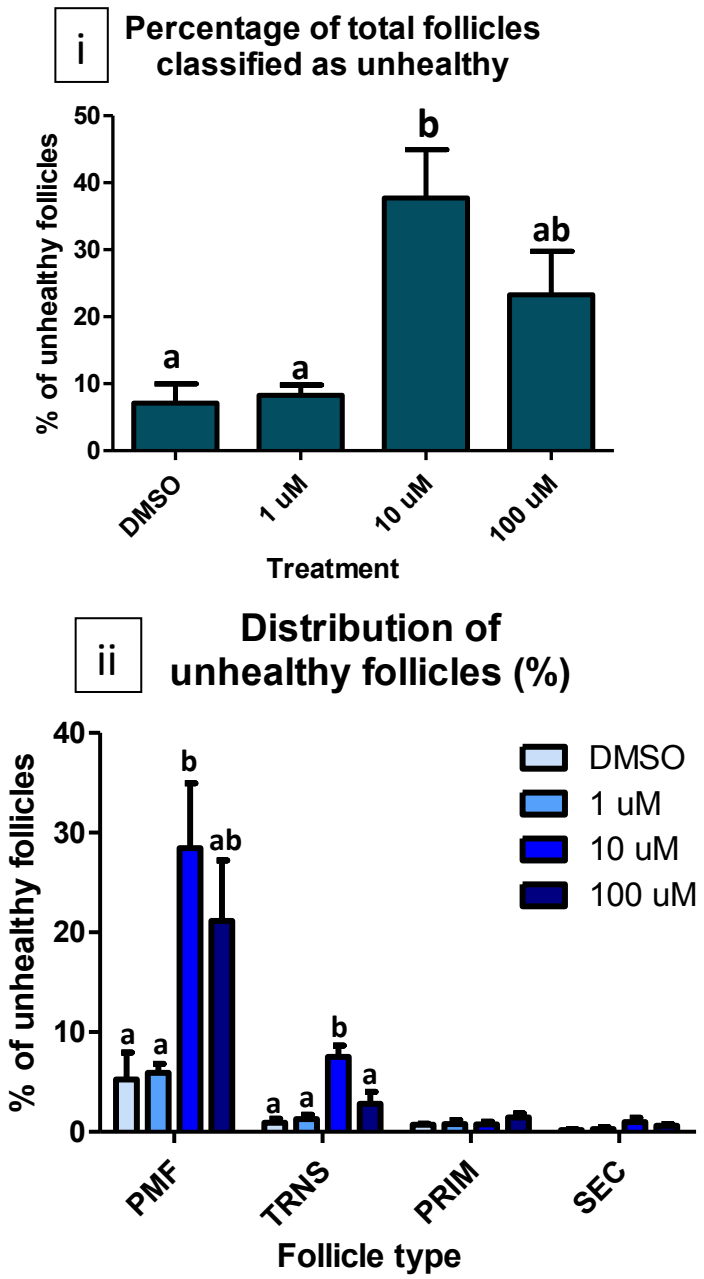


Figure 6.8. Follicle health within AZTC-exposed CD-1 P4 ovaries. There was a significant increase in the percentage (p=0.004) of unhealthy follicles at the medium dose when compared with control and low dose ovaries (i). There was a significant increase in the percentage (p=0.010) of unhealthy primordial follicles at the medium dose compared with control and low dose ovaries. There were also significantly more unhealthy transitional follicles at the medium dose when compared with all other treatments (p=0.0002). No effect was found on the percentage of unhealthy primary (p=0.355) or transitional (p=0.354) follicles (ii). Bars denote mean + sem; n=4 for all groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different (p<0.05).

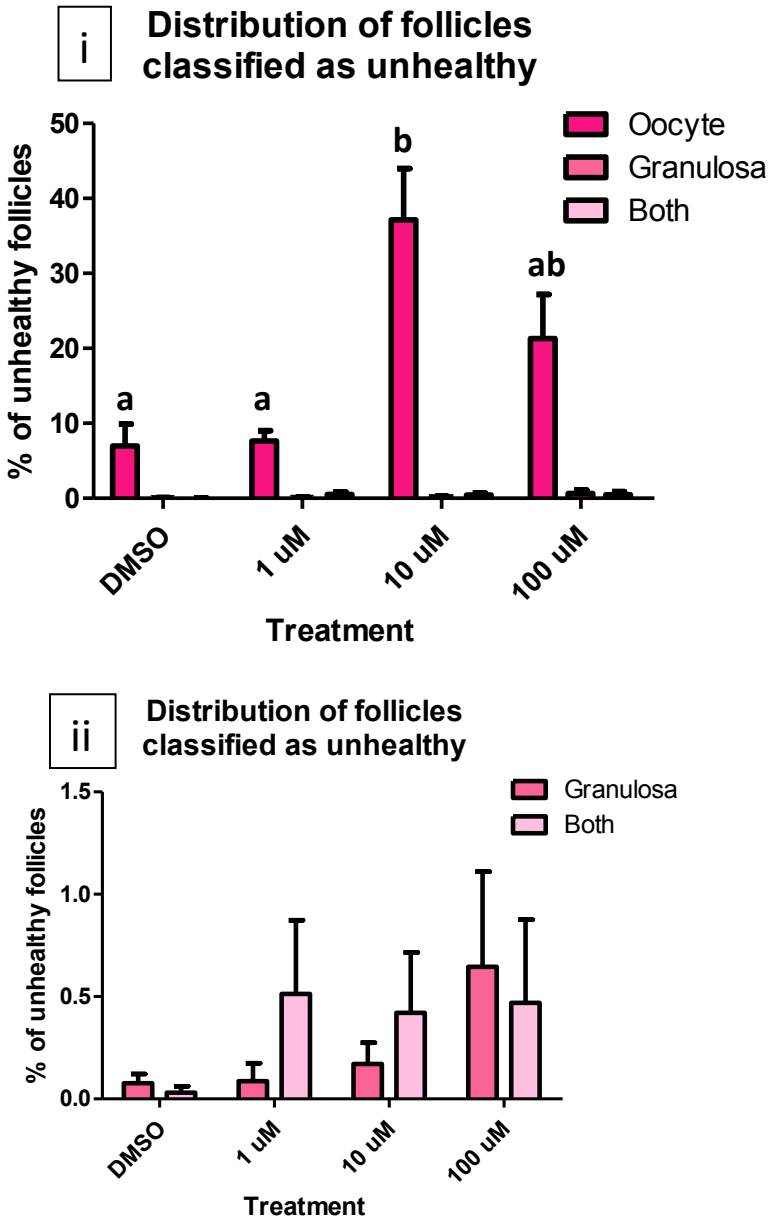


Figure 6.9. Distribution of follicles classified as unhealthy due to an unhealthy oocyte, granulosa cells or both. Unhealthy follicles were further classified into damaged cell types, with the vast majority of follicles being classified as morphologically unhealthy due to an unhealthy oocyte. Furthermore, there was a significant increase in the number of unhealthy follicles due to an unhealthy oocyte at the medium dose when compared with control and low dose ovaries ($p=0.006$) (i). AZTC had no significant effect on the proportion of follicles classified as unhealthy due to unhealthy granulosa cells or both (granulosa: $p=0.338$, both: $p=0.680$). (ii). Bars denote mean + sem; n=4 for all groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different ($p<0.05$).

6.4.3. Experiment 3: Neonatal (P0, CD-1) cultures with etoposide

Etoposide was selected as a secondary study compound due to its known Topo II-inhibitory properties (Section 5.1.1). If similar effects were observed in ovary cultures with etoposide as were observed with AZTC, then this would suggest that AZTC also acts by inhibiting Topo II. However, if the results were different, it would either mean that AZTC does not interfere with Topo II directly, or that it does so by a different pathway than etoposide.

An initial dose-response study had been previously carried out on cultured neonatal (P0) ovaries to assess the appropriate doses of etoposide to use in culture (Section 5.3.5). Following the dose-response study, final concentrations of 50, 100 and 150 ng/ml were selected for future experiments (n=5 for high dose ovaries, n=4 for all other treatment groups). These doses were selected as they were lower than the doses that resulted in complete follicle atresia but were also lower than the range of serum concentration measured in patients following etoposide treatment (5-60 μg/ml) (Section 5.1.1).

Post-natal *in vitro* exposure to etoposide had no significant effect on follicle numbers within the ovaries, although a dose-dependent, but not-significant, reduction in follicle numbers was observed with increasing dose of etoposide (p=0.249) (Fig. 6.10i). Etoposide had no significant effect on the proportion of primordial, transitional, primary or secondary follicle types (Fig. 6.10ii). Upon analysing follicle health within the exposed ovaries, there was a slight, dose-dependent increase in the percentage of unhealthy follicles observed with increasing etoposide dose, although this did not reach statistical significance (p=0.216) (Fig. 6.11i). Similarly, etoposide had no effect on the distribution of unhealthy follicle types within cultured ovaries (Fig. 6.11ii).

In order to determine the cell type responsible for the follicles being classified as unhealthy, and to determine which cell type was being targeted by etoposide, follicles were further classified into 'unhealthy follicle due to an unhealthy oocyte', 'unhealthy follicle due to unhealthy granulosa cells' or 'unhealthy follicle due to both

unhealthy oocyte and granulosa cells'. Nearly all the unhealthy follicles within the post-natally treated etoposide ovaries were classified as such due to an unhealthy oocyte (Fig. 6.12). There appeared to be a dose-dependent increase in the proportion of follicles classified as unhealthy due to unhealthy oocytes, but this was not significant ($p=0.289$).

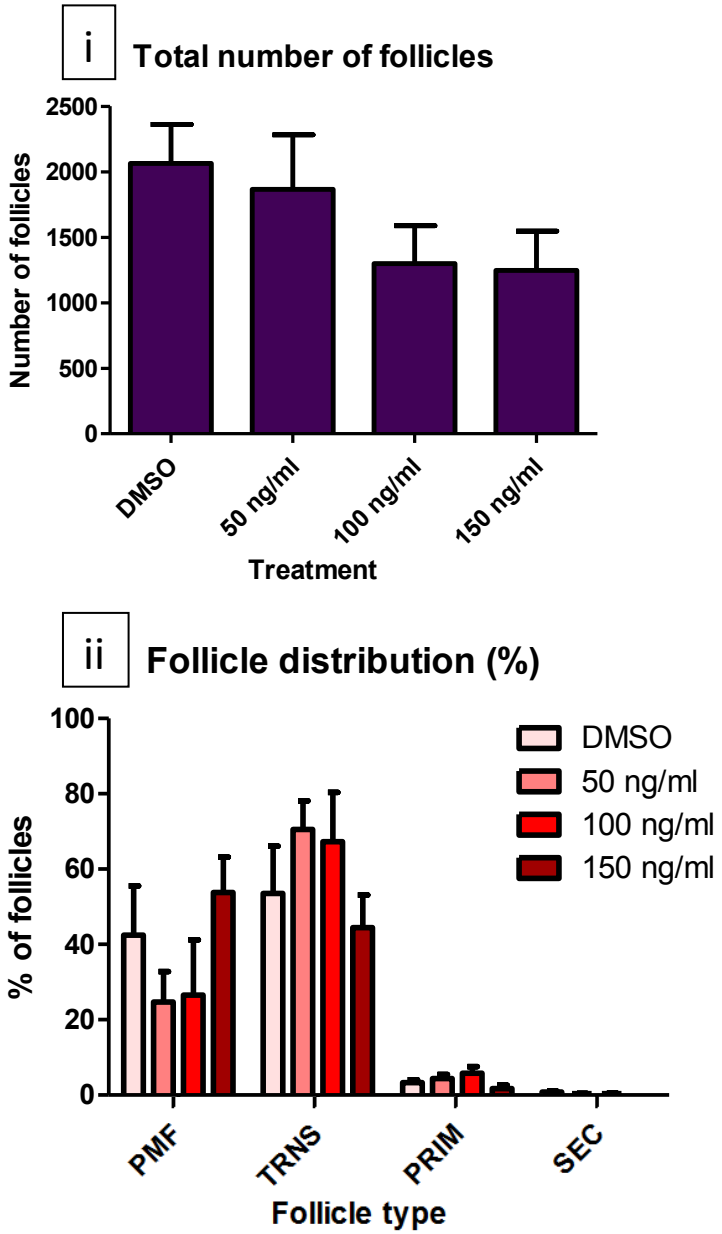


Figure 6.10. Total number (i) and distribution (ii) of follicles within P0 CD1 ovaries exposed to etoposide *in vitro*. No significant effect of etoposide was observed on the follicle number within the post-natal ovaries treated with etoposide (p=0.249). There did appear to be a dose-dependent reduction in follicle numbers with increasing etoposide dose, although this was not significant (i). No significant effect was found on the percentage of any of the follicle types (PMF: p=0.250, TRNS: p=0.288, PRIM: p=0.129) except secondary follicles, where a significant reduction in the percentage of secondary follicles was observed at the high dose (p=0.048), but this was not supported by post-hoc tests (ii). Bars denote mean + sem; n=5 for high dose, n=4 for all other groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary.

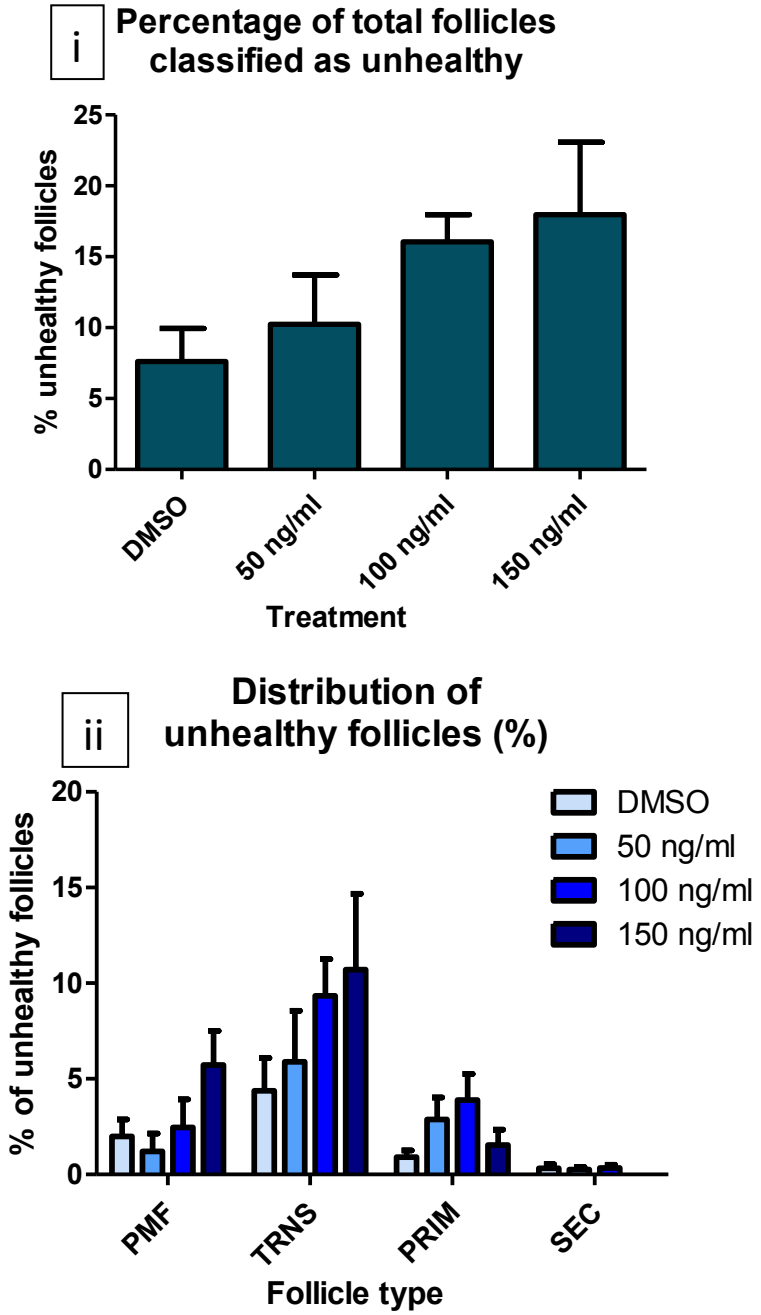


Figure 6.11. Follicle health within P0 ovaries treated with etoposide *in vitro*. Etoposide had no significant effects on the percentage of unhealthy follicles within ovaries (p=0.216). There was a slight, dose-dependent increase in the percentage of unhealthy follicles with increasing etoposide dose, although this was not significant (i). There appeared to be dose-dependent trends for an increase in the percentage of unhealthy primordial and transitional follicles with increasing dose, but this did not reach significance (PMF: p=0.142, TRNS: p=0.415) (ii). Bars denote mean + sem; n=5 for high dose, n=4 for all other groups. Follicle types: PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary.

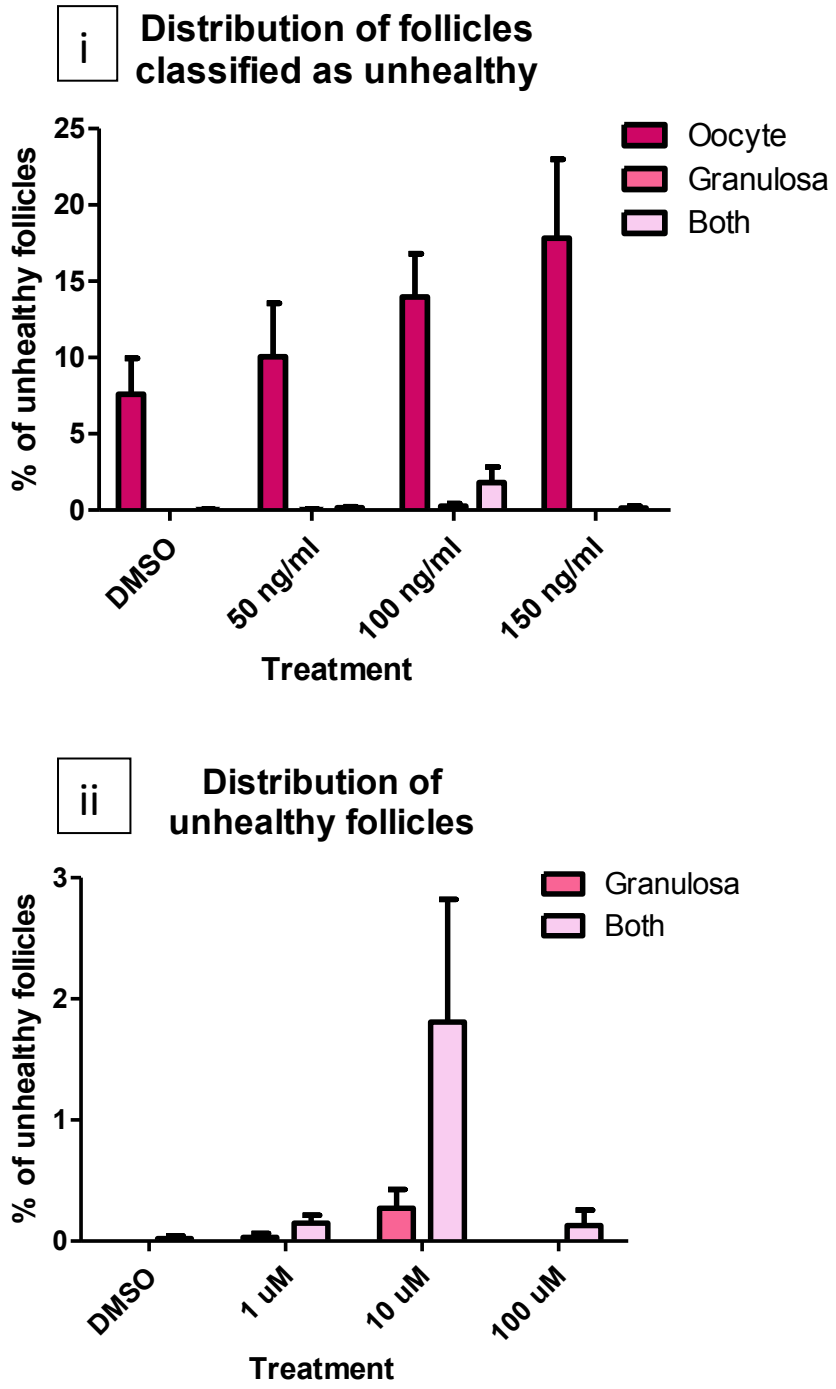


Figure 6.12. Distribution of follicles classified as unhealthy due to an unhealthy oocyte, granulosa cells or both. Unhealthy follicles were further classified into damaged cell types, with the vast majority of follicles being classified as morphologically unhealthy due to an unhealthy oocyte (i). A very small proportion of follicles were classified as unhealthy due to unhealthy granulosa cells (ii). Bars denote mean + sem; n=5 for high dose, n=4 for all other groups

6.4.4. Experiment 4: Newborn ovary cultures with AZTC (F1)

The first cultures carried out for this work had been where newborn F1 ovaries had been cultured with either DMSO or AZTC at 1, 10 or 100 μM concentrations. These cultures were then repeated with CD-1 ovaries, to allow a more direct comparison with the pre-natal cultures (carried out using CD-1 ovaries, see Chapter 5). None-the-less, it seems worth including the results from the F1 experiment here, since they provide an interesting observation on a topic that is often ignored in reproductive biology: inter-strain differences.

At the end of the 6 day culture, no significant effect was observed on the total number of follicles within the ovaries. There did appear to be a slight, dose-dependent reduction in follicle number with increasing AZTC dose, although this was not statistically significant ($p=0.148$) (Fig. 6.13i). When follicles were distributed into the different follicle types, there was a significant decrease in the percentage of primordial follicles at the high dose when compared with all other treatment groups ($p=0.0007$), as well as a significant increase in the percentage of transitional follicles at the high dose ($p=0.0006$). There was no effect on the percentage of primary follicles within treated ovaries ($p=0.333$) (Fig. 6.13ii).

When follicle health was examined, AZTC had an effect on the proportion of follicles within the ovaries that were unhealthy ($p=0.0009$), where the percentage of unhealthy follicles increased significantly at the highest dose when compared with controls, low and medium dose treatment groups (Fig. 6.14i). When the distribution of unhealthy follicles was examined, a significant increase in the proportion of unhealthy transitional follicles was observed within high dose ovaries ($p<0.0001$). Similarly, there were slight increases in the proportions of unhealthy primordial and primary follicles but these did not reach statistical significance (primordial: $p=0.399$, primary: $p=0.064$) (Fig. 6.14ii).

In order to determine the cell type responsible for the follicles being classified as unhealthy, and to determine which cell type was being targeted by AZTC, follicles were further classified into unhealthy due to the oocyte only, granulosa cells only, or

both. The majority of follicles within the AZTC treated embryonic ovaries were classified as unhealthy due to an unhealthy oocyte, although there was a slight, non-significant increase in the proportion of follicles classified as unhealthy due to unhealthy granulosa cells ($p=0.114$). Furthermore, the proportion of follicles classified as being unhealthy due to an unhealthy oocyte significantly increased at the highest dose of AZTC ($p=0.0006$) (Fig. 6.15).

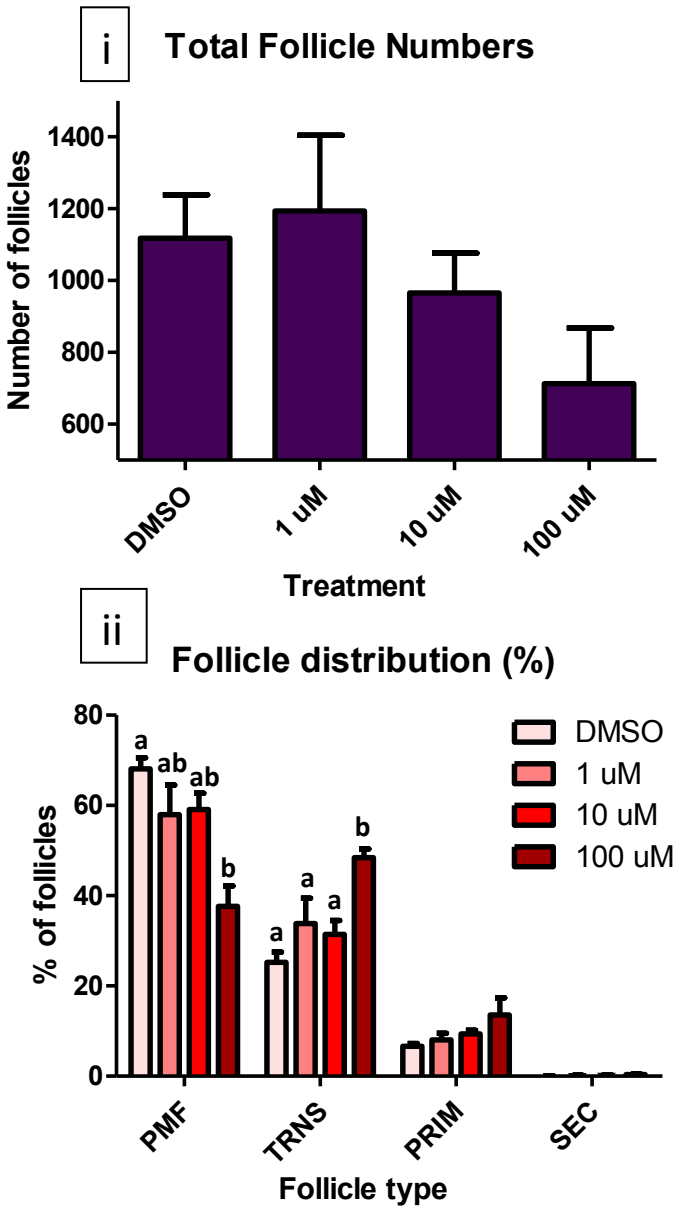


Figure 6.13. Total number (i) and distribution (ii) of follicles within F1 AZTC-treated ovaries. AZTC had no significant effect on the total follicle number within the F1 exposed ovaries compared with controls (p=0.148). A non-significant trend was however observed where exposed ovaries appeared to have slightly fewer follicles with increasing dose of AZTC (i). A significant decrease in the percentage of primordial follicles (p=0.003), and a significant increase in the percentage of transitional follicles was observed at the high dose (p=0.0006), but there were no significant differences in the percentage of primary (p=0.180) or secondary (p=0.333) follicles between treated and control ovaries (ii). Bars denote mean + sem; n=6 for controls, 1 and 10 µM treatment groups, n=7 for the 100 µM treatment group. PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different ($p<0.05$). Means with different letters are significantly different ($p<0.05$).

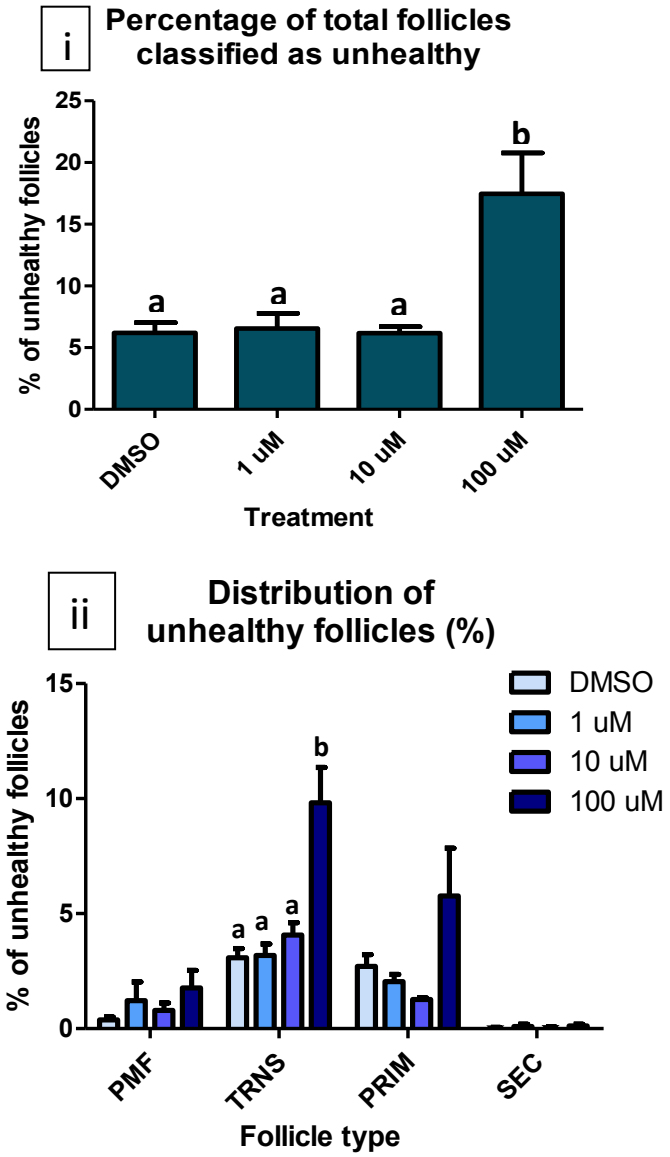


Figure 6.14. Follicle health within F1 AZTC exposed ovaries. The percentage of unhealthy follicles within the high dose ovaries was significantly higher than controls, low and medium dose groups ($p=0.0009$) (i). The percentage of unhealthy transitional follicles in the high dose ovaries also increased significantly ($p<0.0001$), where a slight increase in the percentage of unhealthy primary follicles was observed as well, but this did not reach statistical significance ($p=0.064$). There was no effect on the percentage of primordial follicles in AZTC-treated F1 ovaries ($p=0.399$) (ii). Bars denote mean + sem; n=6 for controls, 1 and 10 µM treatment groups, n=7 for the 100 µM treatment group. PMF: primordial, TRNS: secondary, PRIM: Primary, SEC: secondary. Means with different letters are significantly different ($p<0.05$). Means with different letters are significantly different ($p<0.05$).

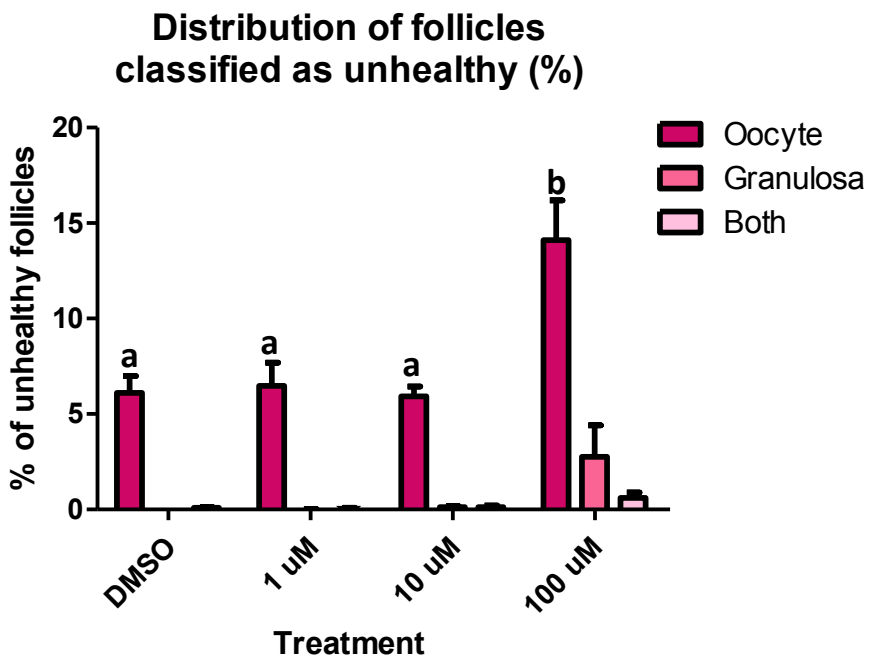


Figure 6.15. Distribution of follicles classified as unhealthy due to an unhealthy oocyte, granulosa cells or both. Unhealthy follicles were further classified into damaged cell types, with the vast majority of follicles being classified as morphologically unhealthy due to an unhealthy oocyte. There was a significant increase in the proportion of follicles classified as unhealthy due to an unhealthy oocyte ($p=0.0006$). There was also a slight increase in the number of follicles classified as unhealthy due to unhealthy granulosa cells or both an unhealthy oocyte and unhealthy granulosa cells at the high dose, although this was not significant (Granulosa: $p=0.114$, Both: $p=0.066$). Bars denote mean + sem; n=6 for controls, 1 and 10 μM treatment groups, n=7 for the 100 μM treatment group. Means with different letters are significantly different ($p<0.05$).

6.5 Discussion

6.5.1 The expression pattern of Topo IIα within the cultured neonatal mouse ovary.

Immunohistochemical detection of Topo IIα was carried out on cultured newborn (P0) ovaries. The expression pattern of Topo IIα in the *in vivo* mouse ovary had been examined previously (Section 5.4.1). Although most germ cells were no longer expressing Topo IIα during the first two days of culture, expression was still found in a few germ cells at this point. Around the third day of culture Topo IIα expression was found in some of the surrounding somatic cells with no more germ cells expressing Topo IIα. By the end of culture, expression was found in many granulosa cells of growing follicles as well as within the stroma, with no germ cells expressing Topo IIα at this point. This was found to correlate quite closely with what was observed in the *in vivo* ovary at the equivalent stages of development and was therefore an expected result. Topo IIα expression had previously been shown to change from within the germ cells to the surrounding granulosa and stromal cells after birth. This did mean, however, that the exposure window of the newborn ovary culture (P0) did start before all Topo IIα expression was restricted to the somatic cells, and therefore AZTC could still have been directly affecting the oocytes at this point, making it difficult to efficiently compare pre- and post-natal AZTC exposure. For this reason, the neonatal ovary culture was repeated collecting ovaries at a slightly later time-point (P4).

It is worth noting that Topo IIα expression was still found within some germ cells in the first few days after birth when Topo II is thought to be involved in meiotic and mitotic divisions (Roca, 2009, Nitiss, 2009, Russell et al., 2000, Li et al., 2013) whereas these germ cells are undergoing neither. Meiosis has ceased by birth, where the germ cells have entered meiotic arrest, but they are not undergoing mitosis either. It is possible that this is residual Topo II remaining in the germ cells that are the last to enter meiotic arrest, but it also suggests that there might be another role for Topo II in germ cells that are not directly involved with meiosis or mitosis.

6.5.2. The effect of AZTC on the post-natal ovary *in vitro*

AZTC had no significant effects on the total number of follicles within cultured newborn (CD-1 P0) ovaries, although there were trends for a dose-dependent decrease in the percentage of primordial follicles with increasing AZTC dose, but this was not significant. The same trend was observed the P4 cultured ovaries, where a non-significant reduction in follicle number was observed in all AZTC-treated ovaries. Furthermore, while there was no significant effect on the distribution of follicles within treated ovaries in cultured P0 ovaries, a significant decrease in the proportion of primordial follicles was observed at the medium dose in P4 cultured ovaries. This corresponded with a significant increase in the proportion of transitional follicles, but again, only up-to the medium dose.

The pattern of follicle health was also fairly consistent between exposed P0 and P4 ovaries, where overall, follicles appeared to be more unhealthy at the medium dose than at the top dose of AZTC. There was a slight non-significant increase in the percentage of unhealthy follicle numbers up-to the medium dose in P0 ovaries, reaching significance in the P4 ovaries. The percentage of unhealthy follicles then dropped again at the highest dose in both P0 and P4 cultured ovaries. When the distribution of unhealthy follicles was analysed, a similar pattern was consistently observed in both P0 and P4 ovaries. In P4 ovaries there was a significant rise in the proportion of unhealthy primordial and transitional follicles up to the medium dose, dropping off at the highest dose. In P0 ovaries, the same trend was observed for both primordial and transitional follicles, but only reached significance for the transitional follicles. The oocyte appeared to be the primary target for AZTC in both P0 and P4 cultured ovaries, with very few follicles classified as unhealthy due to unhealthy granulosa cells. This result was somewhat surprising, as AZTC was expected to inhibit dividing cells, such as granulosa cells, in particular because Topo IIα is expressed within the granulosa and stromal cells in the post-natal ovary. Even more surprisingly, the effect on oocytes only reached significance in the P4 ovary culture but not in the P0 culture, when the P0 culture encompasses the tail-end of Topo IIα expression within the oocytes, whereas the P4 ovary culture was chosen to avoid any Topo IIα being expressed within the oocytes.

6.5.3. How does exposure of the post-natal ovary to AZTC differ from pre-natal exposure?

AZTC had previously been shown to affect the pre-natal ovary, where it resulted in an increase in follicle number at lower doses, followed by a reduction at the high dose. An increase in the number of unhealthy follicles was also observed both *in vivo* (at the high dose) and *in vitro* (at low and medium doses only) (Sections 3.4.2 and 5.4.2, respectively). In the post-natal *in vitro* ovary, however, the initial increase in follicle number was not observed, only a decrease in the number of primordial follicles (See Appendix D). The distribution of unhealthy follicles correlated fairly closely between pre- and post-natal exposure ovaries, where the medium dose (10 μM) resulted in a higher proportion of unhealthy follicles than the high dose (100 μM). This suggests that the effects of AZTC apply to both the pre- and post-natal ovary, where it results in a reduction of the size of the follicle pool and increased number of atretic follicles. This is further supported by the complete lack of follicles at the highest tested doses of AZTC *in vitro* (200 μM in the embryonic ovary culture, and 500 μM in embryonic and neonatal ovary cultures).

6.5.4. Effect of etoposide on the neonatal mouse ovary.

Etoposide resulted in a dose-dependent reduction in follicle numbers in the cultured neonatal ovary, although this was not significant. Similarly, no significant effect was found on the proportion of unhealthy follicles within these cultured ovaries. Furthermore, somewhat surprisingly, it was the oocyte that was the primary target of etoposide. There was also a slight increase in the proportion of oocytes categorised as unhealthy due to unhealthy oocytes and granulosa cells, but this was a very small proportion of follicles.

The post-natal *in vitro* ovary did not appear to be as susceptible to ovary damage following etoposide exposure, unlike the pre-natal *in vitro* ovary (Section 5.4.2). The embryonic mouse ovary cultured with etoposide had in an increase in the number of unhealthy follicles and a significant reduction in the size of the follicle pool. Although a slight reduction was observed in the follicle pool of the post-natal ovary, this did not occur to the same extent as in the pre-natal ovary, and was not

significant. Furthermore, although a slight increase in the proportion of unhealthy follicles was recorded in the post-natal ovary, again this was not significant. This result was not unexpected, as it had been suggested previously that the post-natal ovary is less susceptible to etoposide damage than the pre-natal ovary, although the evidence for the reproductive toxicity of etoposide in the adult ovary has been conflicting (Oktem and Oktay, 2007, Choo et al., 1985). It is possible that once follicles have formed and oocytes are no-longer undergoing meiosis, that they become less susceptible to etoposide-induced damage. This could further be supported by the fact that Topo IIα is no longer expressed in the oocytes of the post-natal ovary. Etoposide was, however, expected to elicit effects on granulosa cells which are undergoing divisions and expressing Topo IIα during post-natal ovary development, but no effect on granulosa cells was observed here. It is possible that the doses selected were too low for the post-natal ovary, where the pre-natal ovary may be more susceptible to damage at doses lower than what would result in ovary damage in the post-natal ovary. Furthermore, the number of ovaries for each treatment group was perhaps slightly too low, where the study would most likely have benefitted from having at least 6 ovaries per study group as there can be a lot of variability in follicle numbers and health between different ovaries, in particular CD-1 mice which have a very high number of follicles in the ovary compared with other mouse strains (Canning et al., 2003). In the future, it would be interesting to repeat the study using both P0 and P4 ovaries, including a higher number of ovaries per treatment group. It would also be possible to add another treatment group of a higher dose of etoposide, 200 μg/ml for example, which is still lower than the recorded serum levels following etoposide exposure.

6.5.5. Mechanisms of action of AZTC

Various different experiments were carried out covering *in vivo* and *in vitro* exposures of AZTC, as well as pre- and post-natal exposure windows of AZTC and etoposide, covering the time that Topo IIα is either expressed within germ cells or within the granulosa and stromal cells. This was done to investigate if different effects might be observed when AZTC exposure occurred at a time when Topo IIα was expressed within germ cells compared with when it was expressed within

somatic cells. If AZTC was acting by inhibiting mammalian Topo IIα, we would have expected to observe different results between pre- and post-natal exposure ovaries. The results between the embryonic, P0 and P4 cultures show fairly similar patterns, with a rise in unhealthy follicles up-to the medium dose, and with AZTC targeting mainly the oocytes. It is difficult to draw conclusions about the actions of AZTC from the data available, since it is still possible that AZTC elicits effects on the ovary by an off-target effect that is not necessarily a result of direct mammalian Topo II inhibition. On the other hand, considering the similarities between the effects of etoposide and AZTC, there could also be effects on the granulosa cells in the post-natal ovary, which in-turn, result in secondary effects on the oocytes. This will be discussed further in Chapter 7.

6.5.6. Inter-strain differences in AZTC-exposure between F1 and CD-1 mouse ovaries.

Initially, ovaries from newborn F1 mice had been used to investigate the effect of AZTC on the post-natal ovary. Due to the relatively small litters born to F1 mothers, CD-1 mice had then been selected to develop the embryonic ovary culture, due to considerably larger litters born to CD-1 mothers. Therefore, the newborn (P0) ovary culture was repeated with CD-1 mice to allow direct comparisons of ovarian effects of AZTC between the pre- and post-natal ovary. However, the initial use of F1 mice in these cultures meant that comparisons could be made between the two strains and their responsiveness to AZTC.

At first, F1 and CD-1 control ovaries were compared to investigate if there were any underlying differences between the two strains. The main difference was that CD-1 mice contained considerably more follicles (around 1600) than the F1 ovaries (around 1100). Furthermore, the distribution of follicles was different between the two species, where CD-1 ovaries had more primordial follicles (around 88%) per ovary, compared with the F1 ovaries (around 73%). In turn, F-1 ovaries contained slightly more transitional follicles (around 25%) when compared with CD-1 ovaries (around 13%), and primary follicles, but only by a relatively small amount (primary follicles: around 6% in F1 vs. 4% in CD-1). This suggests that F1 ovaries have a

slightly higher rate of follicle recruitment than do CD-1 ovaries. These results were consistent with what was expected since CD-1 mice have previously been described as having a greater number of follicles within their ovaries than many other strains of mice (Canning et al., 2003).

It was then examined whether the two strains differed in the way they were affected by AZTC. Several similarities were observed between the strains following neonatal ovary exposure to AZTC. The main similarities were that they both had a slight (non-significant) decrease in total follicle numbers and a dose-dependent decrease in the number of primordial follicles (significant in F1 ovaries, not-significant in CD-1 ovaries). That aside, the results appeared to vary somewhat between the two strains. Firstly, while there was a significant dose-dependent reduction in the percentage of primordial follicles, correlated with a significant increase in the percentage of transitional follicles in the F1 ovaries, AZTC had no effect on the percentage of primordial or transitional follicles in CD-1 treated ovaries. When the total numbers of each follicle type per ovary were compared however, there were more similarities visible, where primordial follicles were reduced in both strains, but only reached significance in F1 ovaries. Secondly, in general, the F1 ovary was consistently more affected by the top dose of AZTC than were CD-1 ovaries. In CD-1 ovaries, more unhealthy follicles were found at the medium dose than at the top dose, with the proportion of unhealthy transitional follicles significantly increased at the medium dose. In F1 ovaries however, this did not occur until at the high dose. A similar effect was observed on total numbers of unhealthy follicles, where there was a significant increase at the top dose of AZTC in F1 ovaries, but only a slight non-significant increase in the number of unhealthy follicles up-to the medium dose in CD-1 ovaries. This effect was again repeated when analysing the proportion of follicles classified as unhealthy due to unhealthy oocytes.

These results were unexpected as, despite the differences in total follicle numbers and slight differences in the distribution of follicles between the two strains, AZTC was expected to affect ovaries in a similar way regardless of the strain. Although there were some similarities between the strains, it is possible that due to a smaller

pool of follicle to begin with, any effects on F1 ovaries might result in a higher statistical significance than in the CD-1 ovary that contains a larger follicle pool. That is, the large number of follicles within CD-1 ovaries might in some ways 'mask' effects of AZTC if only a proportion of the follicles are affected by the compound. Furthermore, even if a relatively small proportion of follicles within F1 ovaries are affected and become atretic, other healthy primordial follicles may exit the resting pool of follicles to grow, since follicles act by inhibiting each other from entering the growing pool (Kalich-Philosoph et al., 2013). The smaller the follicle pool, the more likely the quiescent follicles are to grow and undergoing atresia. In CD-1 ovaries on the other hand, the effects may not be observed until much later, due to the larger pool of primordial follicles to start off with, resulting in increased inhibition of follicle activation. Another possibility is that F1 mice were more susceptible to ovarian damage by AZTC than CD-1 mice. This could be due to genetic differences between the strains. CD-1 mice have previously been shown to have reduced sensitivity to estrogen, where there were large variations in ovarian responsiveness to gonadotropins between different strains. As a consequence, the ovaries of CD-1 mice are far more resistant to endocrine disruptors than other strains of mice (Spearow et al., 1999, Spearow et al., 2001). If AZTC is acting by having a non-Topo II (off-target) effect, or potentially affecting E2 within these ovaries, it would not be surprising that CD-1 ovaries were less affected by AZTC. These results raise the importance of investigating inter-species strain differences in mice commonly used in the laboratory, as this is a topic that is not often raised or investigated.

6.6. Conclusions

Neonatal P0 and P4 ovaries cultured with AZTC demonstrated consistently similar effects, namely a dose-dependent decrease in total follicles, with significant decreases in primordial follicle numbers, and an increase in unhealthy follicles up-to the medium dose, where AZTC primarily targeted the oocyte. This also corresponded closely with effects observed following pre-natal *in-vitro* AZTC exposure, suggesting that perhaps AZTC could be eliciting its effect via an off-target route, since Topo IIα is expressed within the germ cells pre-natally, but within the granulosa and stromal cells post-natally. Neonatal cultures with etoposide had no significant effects on the post-natal ovary, where the results were somewhat inconsistent with neonatal AZTC exposure. This makes it difficult to directly compare effects of AZTC and etoposide, or to elucidate how AZTC acts on the ovary, without further investigation. Finally, when F1 and CD-1 ovaries were compared, CD-1 mice were found to have a larger pool of follicles and in slightly different ratios to F1 ovaries. The two strains differed in their responses following AZTC exposure, where F1 ovaries were affected by AZTC to a much greater extent than CD-1 ovaries. This could be due to various reasons that include genetic differences between the strains, their susceptibility to damage following exposure to gonadotropins and/or ovotoxicants, or the underlying differences in follicle numbers.

6.7. Future directions

In the future, it would be interesting to carry out tests to investigate if AZTC is inhibiting Topo II, and if so, by what mechanism. That is, if AZTC inhibits Topo II in a way that reduces the level of the protein present within the cell, a Western Blot could be carried out following AZTC exposure and AZTC protein levels would be expected to decline with increasing AZTC exposure. On the other hand, if AZTC acts by blocking or inhibiting the action of the enzyme, then the protein would still be expected to be present within the cells but would instead have been rendered inactive, in which case the protein levels would not be expected to reduce. The other possibility is, of course, that AZTC does not act on mammalian Topo II, in which case we would also see no change in protein levels. It would also be useful to carry

out further investigations into the effects of AZTC and etoposide exposure during meiosis I by repeating the chromosome spreads, or on the meiotic resumption of an ovulated and fertilized oocyte. Etoposide has previously been suggested to cause defects in chromosome condensation and segregation in mouse oocytes, although Topo II has also been suggested to be dispensable for meiotic resumption (Li et al., 2013). This could potentially outline further whether the effects of AZTC and etoposide are more pronounced during meiosis, as so far, they appear to have more pronounced effect on the pre-natal ovary than on the post-natal ovary. Finally, another study that could be carried out would be to investigate if AZTC and etoposide affect steroid production in the growing ovary by carrying out estradiol assays on the culture medium as this would be an important effect if replicated *in vivo*.

Chapter 7.
Discussion

7.1. Summary of Results

There is increasing evidence to indicate that a substantial number of both man-made and naturally occurring chemicals are disruptive to human and wildlife reproductive health. One period that is considered particularly vulnerable is that of pre-natal ovary development, during which meiosis I occurs. Current reproductive toxicology testing is primarily carried out *in vivo*, although in the past half a century, a diverse range of culture methods have been developed culturing oocytes or whole ovaries at different time-points of development. Very few of the established embryonic ovary culture systems, however, have been used to investigate potential reproductive toxicants on the pre-natal ovary. This aim of this project was to set up and develop such an embryonic ovary culture system that would span early meiosis, germ cell nest breakdown, follicle formation and growth initiation. The culture system could then potentially be used to study the dynamics of early ovary development, as well as to assess the reproductive toxicology of man-made and environmental chemicals.

The novel embryonic ovary culture system was developed by adapting, improving and bridging pre-existing available culture techniques. The culture system set up here supported early meiosis of cultured oocytes, through prophase I, to the formation of primordial follicles and finally, initiation of follicle growth to the primary follicle stage. Various methodologies were attempted to achieve this, including modifications to the culture medium, culture techniques and the inclusion or exclusion of the mesonephros prior to culture. The culture method that produced the healthiest looking follicles with visible basal lamina, consisted of a 12 day culture on an agar block, where ovaries were cultured for 3 days in complex medium, followed by 9 days in simple medium, with the mesonephros attached for the duration of culture. Histological analysis of cultured ovaries revealed that they were morphologically normal and healthy, and that they contained follicles at stages in comparable ratios as *in vivo* ovaries. The presence of basal lamina was further examined using a biotin tracer study, which revealed the appropriate establishment of basal lamina in most follicles, although the cultured ovaries showed less structural organisation than did *in vivo* ovaries. Finally, chromosome spreads were carried out on cultured embryonic ovaries to investigate whether the culture system supported

the progression of oocytes through prophase I of meiosis. The chromosome spreads revealed that homologous chromosomes in cultured oocyte nuclei were capable of progressing through leptotene, zygotene, and fully synapse at the pachytene stage of meiosis in an identical manner to *in vivo* oocyte nuclei. The cultured ovaries were, however, slightly delayed when compared with *in vivo* ovaries.

A novel test compound, a topoisomerase II inhibitor: AZTC, was then used to assess the efficacy and validity of the ovarian culture methods. This was done by comparing embryonic mouse ovaries that had been cultured with AZTC during prophase of meiosis I, with *in vivo* studies where rat embryos were exposed to AZTC during gestation. AZTC was selected as a study compound due to its detrimental effects on spermatogenesis in male rats. AZTC targets bacterial Topo II but it was presumed to act on mammalian Topo II as well, due to the close homology between bacterial Topo II and mammalian Topo II. Topo IIα was expressed within the female germ cells pre-natally, but became localised to the granulosa and stroma cells post-natally. This occurred both *in vivo* and *in vitro.*

Embryonic ovaries cultured with the low and medium dose of AZTC consistently demonstrated similar effects to that observed in the *in vivo* pre-natal AZTC-exposed rat ovary. On the other hand, AZTC demonstrated different effects when exposure occurred pre-natally vs. post-natally. Furthermore, another Topo II inhibitor, etoposide, was used as secondary test compound, to assess the validity and efficacy of the embryonic ovary culture. Etoposide-treated embryonic ovaries had a reduction in the size of the follicle pool, with primordial and transitional follicles targeted.

The various different experiments carried out on AZTC and etoposide, on both pre-natal (*in vitro* and *in vivo*) and post-natal (*in vitro* only) are outlined in Table 7.1. This therefore allows for numerous comparisons to be made comparing:

i) *In vivo* vs. *in vitro*
ii) Pre- vs. post-natal effects of AZTC
iii) Effects of etoposide vs AZTC
iv) F1 vs CD-1 strains

	Pre-natal		Post-natal			
	AZTC	Etoposide	AZTC		Etoposide	
			P0	P4	P0	P4
In vitro	CD-1 mice	CD-1 mice	CD-1 & F1 mice	CD-1 mice	CD-1 mice	
In vivo	Wistar rats					

Table 7.1. Outline of all the different experiments carried out on AZTC and etoposide

7.2. Advantages and disadvantages of using *in vitro* testing in reproductive toxicology studies.

The majority of the work presented in this thesis was carried out *in vitro*. Cultures have become a widely used tool in to study the development of follicles in reproductive biology and toxicology.

This topic formed the basis of a review written by me, with some of the text below reproduced directly from that article (Stefansdottir et al., 2014). *In vitro* ovary and follicle culture models allow for the possibility of varying culture parameters in a highly controlled manner, and thus have the potential to allow a more thorough evaluation for reproductive toxicity studies than do *in vivo* studies alone. Considering that animals, including humans, are exposed to a very wide range and number of compounds and chemical mixtures in their lifetime, it is becoming increasingly crucial to develop and improve the *in vivo* assessments and *in vitro* culture techniques necessary to elucidate the toxic effects of potential ovotoxicants on the ovary, to allow for faster screening of potential developmental and/or reproductive toxicants. The key point of *in vivo* studies is to assess the potential toxic risk of a drug on the body, when administered at a therapeutic dose. Although this is more representative of the 'real life' situation, it can also be difficult for *in vivo* studies to assess how much is reaching the gonads, since compounds can be detoxified, activated or eliminated in the body. Calculations to determine the amount of compound reaching the gonads are complex and vary between species and life stage. The method of exposure also needs careful consideration as subcutaneous injections

and oral ingestions of the same amount of the same compound will not necessarily result in the same ovarian exposure (Doerge et al., 2011, Fisher et al., 2011). Other issues reproductive toxicologists face when using *in vivo* studies include attempting to limit the duration of exposure to a single dose of compound, since some compounds can, for example, persist in the animal. Exposure of compounds can change due to mobilization of maternal body reserves during pregnancy (Herreros et al., 2010) or can be passed through breast-milk a long time after the exposure window, making it difficult to predict the precise time and duration of exposure (Miller et al., 2004, Doerge et al., 2010). *In vivo* studies often use end-points such as pregnancy, implantation and number of offspring, which are parameters that do not identify the site of action, the mechanism(s) of toxic damage or the effect on the primordial follicle pool. However, female reproductive function requires effective communication between the ovary, the neuroendocrine system, the hypothalamic-pituitary-gonadal (HPG) axis and the reproductive tract, and *in vivo* studies will be able to detect toxic effects on any of these systems, which could result in a secondary effect on the gonads. For example, effects on the estrogen-dependent endometrium, could subsequently lead to ovary-independent infertility (Suzuki et al., 2002, Zama and Uzumcu, 2010). Therefore, one drawback of *in vitro* studies is that, although they can be useful for assessing direct effects on the ovary, they cannot account for any indirect action that might modulate hormone-signalling pathways such as the HPG axis. Studies carried out *in vitro* are also unable to take metabolism into consideration and care must be taken when examining effects of a compound that has no effect until it has been metabolised. Even if a compound has demonstrated interference with receptor binding/hormone production *in vitro*, the same activity may not be observed in vivo (Munn and Goumenou, 2013). Despite this, *in vitro* models are a promising area in toxicology, allowing pragmatic and mechanistic studies of action of reproductive toxicants and are able to reduce the number of animals required for in vivo studies. *In vitro* systems are proving to be an invaluable preliminary method to investigate direct effects of potentially harmful compounds on the female reproductive system, especially where appropriate care has been taken to administer doses that reflect human exposure levels in at least part of the dose-response curve design. Crucially, they require relatively little time to yield precise

answers, and can cover various, yet specific, stages of ovary and follicle development. *In vitro* studies allow scientists to examine the precise mechanisms of action of a reproductive toxicant on the different stages of growth and development, as well as to pin-point whether a specific chemical targets the stroma, the oocyte, the somatic compartment of the follicle, or the follicle as a whole. Therefore, although *in vitro* cultures are less useful for studying indirect toxic effects on the reproductive system, they still do have great potential to provide an important preliminary or secondary screening protocol for toxicology testing alongside *in vivo* studies. The combination of *in vivo* and *in vitro* work is a powerful one to detect and understand mechanisms of damage to the ovary, its follicles and oocytes, and their consequence for adult fertility and subsequent generations.

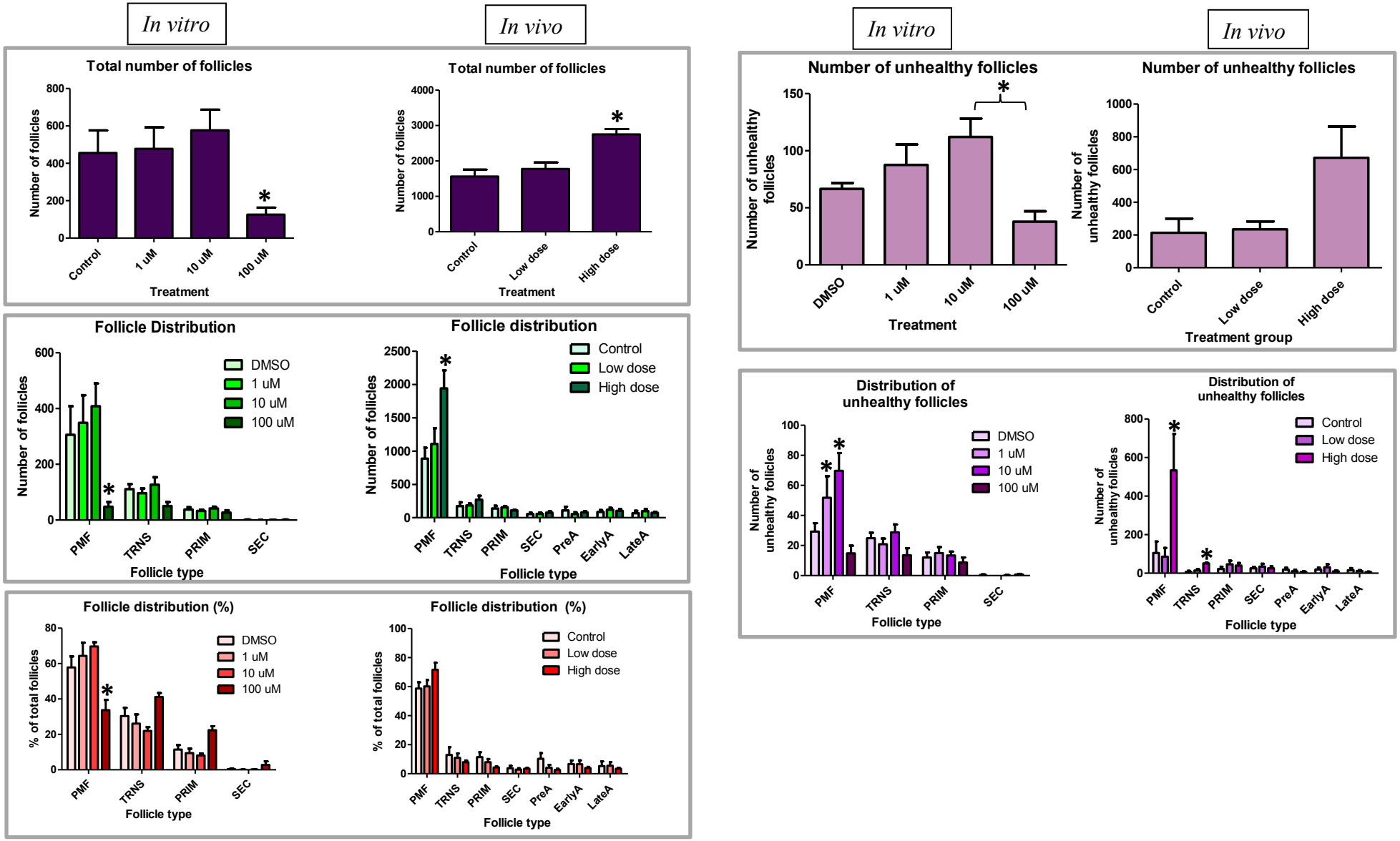
7.3. Comparing *in vivo* vs. *in vitro* results of AZTC on the pre-natal ovary

AZTC was developed to target bacterial type II topoisomerases, given the close homology between bacterial and mammalian Topo II, it is likely that AZTC will also inhibit mammalian homologues that are involved in meiosis. When administered to male rats, it resulted in a detrimental effect on spermatogenesis. The main aim of this project was therefore to examine the effects of AZTC on the ovary, using both *in vivo* and *in vitro* studies, to investigate how *in vitro* cultures might compare with the 'real life' *in vivo* situation. Despite the differences in species between the two studies (rat vs. mouse), the exposure of AZTC occurred during the same crucial time windows: entry into meiosis, prophase I of meiosis up-to meiotic arrest, germ cell nest breakdown and follicle formation. Histological analysis of AZTC-exposed ovaries should therefore reveal any major effects on any of these stages.

Ovaries collected from PND 15 rats exposed to AZTC *in utero*, consistently demonstrated similar results to cultured embryonic ovaries exposed to the low and medium doses of AZTC (Fig. 7.1). In both studies, follicle numbers were slightly increased (significantly so *in vivo)*. This was due to a rise in primordial follicles numbers. Similarly, both *in vivo* and *in vitro* ovaries showed a rise in the number of unhealthy follicles, where there was an increase in the number of unhealthy

primordial follicles. The main difference between the two studies came from the in cultured ovaries exposed to the high dose of AZTC. In that instance, there was a large, significant drop in the size of the follicle pool, along with a drop in the number of primordial follicles, and in the number of unhealthy follicles. This makes it difficult to confidently conclude that the *in vitro* culture correlated with the effects observed *in vivo*. It is possible that the cultured ovaries may be more susceptible to ovarian disruption and damage than *in vivo,* possibly due to the fact that the ovaries have been removed from the ovarian bursa and blood supply. The culture environment may also not provide the same support as the real life situation. On the other hand, it is not known how the *in vivo* doses compare with the *in vitro* ones. The concentration that is reaching the ovaries *in vivo* might be considerably lower than the *in vitro* doses and it is therefore possible that the doses reached *in vivo* are roughly equivalent to the low and medium doses *in vitro,* whereas at doses higher than this (100 µM and above), the follicles are destroyed earlier on in culture, consistent with what is observed at the top doses tested *in vitro* in the dose response study (200-500 µM). Furthermore, there were some *in vivo* ovaries exposed to the high dose of AZTC *in vivo* that contained no follicles and appeared very unhealthy. Although the source of these ovaries could unfortunately not be confirmed, and they were consequently excluded from the study (See Section 3.4.2), it is nevertheless possible that this was a result of AZTC exposure. Finally, it must still be borne in mind that all the results from the *in vivo* PND 15 study came from one female, making that particular result vulnerable to a possible litter-effect, in particular because no effects of AZTC were observed at earlier or later time-points. Bearing this in mind, and since the *in vivo* results consistently correlate with the *in vitro* cultures at low and medium doses of AZTC, it does suggest that AZTC acted on the ovary in a similar manner *in vivo* and *in vitro*. Consequently, it does suggest that the embryonic ovary culture could be a useful tool to determine potential reproductive toxicants as a preliminary screening tool. Furthermore, these cultures could be used as a way to elucidate if chromosomal sensitivity of oocytes to certain compounds or drugs is dependent on meiotic stages.

Figure 7.1. *In vivo* vs. *in vitro* effects of AZTC on the rodent ovary (Sections 5.4.2 &3.4.2, respectively).



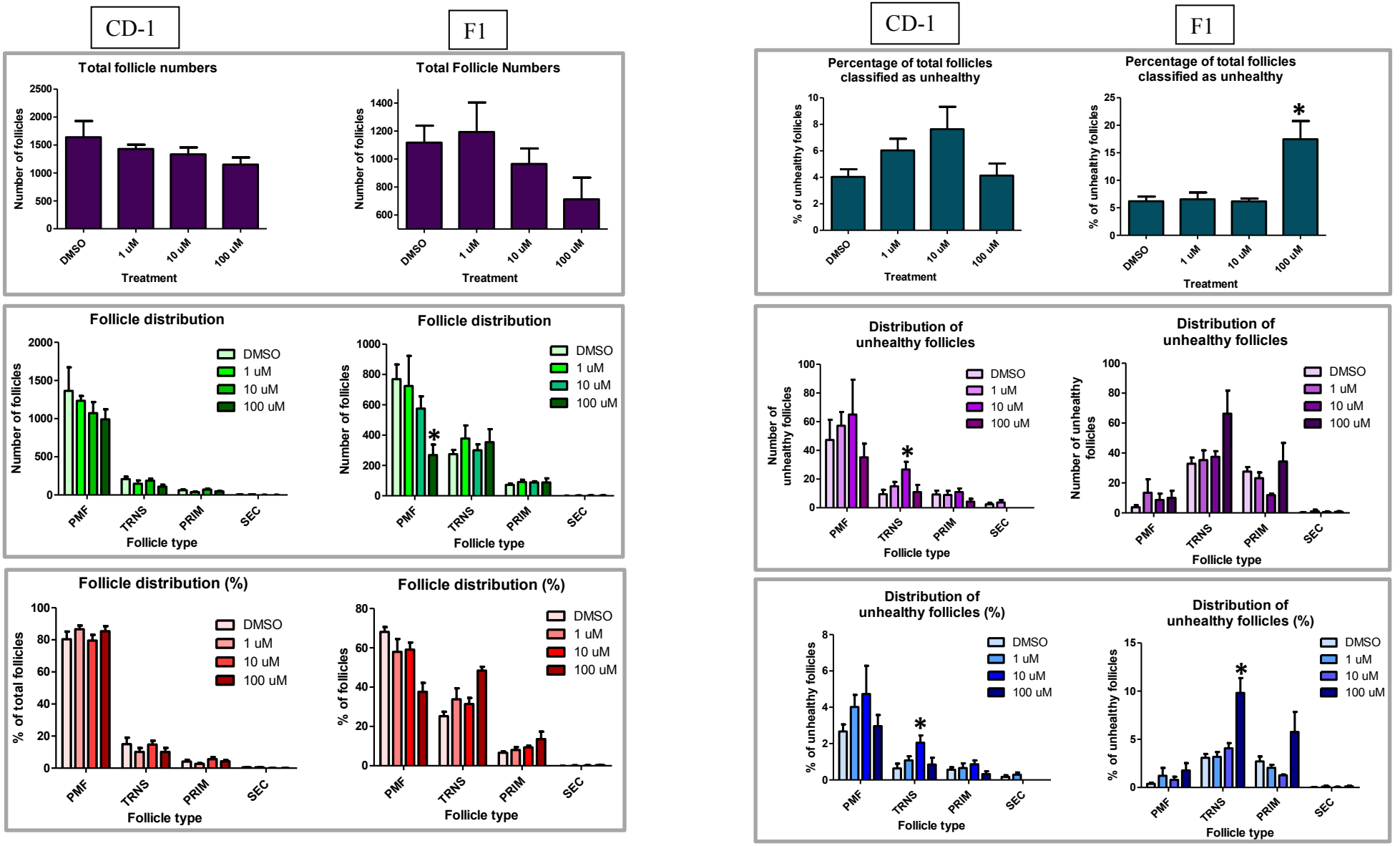
7.4. Inter-strain and inter-species differences.

Analysis of biological experiments must always be carried out with caution, as was demonstrated during this project, strains of the same species can vary in their susceptibility to ovarian disruption following compound exposure. This is, in particular, thought to be the case in mouse susceptibility to disruption of E2 levels within the ovary, where CD-1 mice are less susceptible than other strains such as F1 mice. Here, CD-1 mice also showed more resistance to AZTC than did F1 mice (Fig. 7.2). Inter-strain differences is a frequently ignored subject in science, but the results here further demonstrate the importance of improving our understanding and awareness of these inter-strain differences when it comes to reproductive toxicology, as well as for scientists in general. More efforts should be made to identify and study genetic variations between mouse strains, to further understand underlying genetic differences, and in reproductive biology, to understand differences in their sensitivity to gonadotropins or chemicals, differences in ovarian function as well as their vulnerability to disease. Furthermore, it cannot be excluded that some of the differences observed when comparing the *in vivo* and *in vitro* studies in this project, were due to inter-species differences between the mouse and the rat.

This issue also opens up the discussion about the usefulness of the mouse or rat model as a way to investigate the reproductive toxicity of drugs on the human. The mouse and rat are frequently used models to investigate potential ovotoxicants and have been proven to be invaluable tools for reproductive research. The mouse culture is also the best available model from which live offspring have been produced from cultured primordial follicles (O'Brien et al., 2003). Rodents make a good model due to their short time-course, high material availability, the ease by which they can be genetically manipulated, but also due to their reproductive similarities to women, such as being born with a finite number of follicles. However, it is also important to consider differences and limitations in using the rodent model for reproductive studies. The mouse is polyovular, has a short estrous cycle and has considerably less stromal tissue than the human ovary. Furthermore, there is no certainty that a compound will elicit the same response in the human ovary as in the mouse, in particular since doses can be difficult to correlate between the two species.

Compounds can also not have a typical 'dose-response' curve, where some effects may be missed if the relevant dose is not tested. The drug dosage must also be appropriately translated from one species to the other (Regan-Shaw et al., 2008). Finally, the differences in susceptibility, vulnerability, windows of exposure, genetics and many others must be considered. To give one example of this, it has now become recognised that rodents and humans do not metabolise xenobiotics in the same manner, partly due to the species differences in P450s expression and its catalytic activities (Caldwell, 1981, Bogaards et al., 2000). In contrast, there are also drawbacks to culturing human ovarian tissue, in that it has limited availability and it is not yet a well-established experimental model for reproductive toxicology testing.

Figure 7.2. Comparisons between CD-1 and F1 strains & their ovarian sensitivity to AZTC (Sections 6.4.2.2 & 6.4.4, respectively)



7.5 Elucidating a possible mechanism of action for AZTC

The various cultures and *in vivo* studies carried out throughout the course of the project, aimed to not only validate the embryonic ovary culture system but also to elucidate how AZTC might be affecting the ovary. From analysing all these studies, there are various possibilities to consider (Table 7.1). In some cases, the results suggested that AZTC might act directly by inhibiting mammalian Topo II, whereas other studies suggested that this was not the case. In the pre-natal ovary culture and the *in vivo* study for example, AZTC resulted in an initial increase in follicle numbers, followed by a great drop in follicle numbers *in vitro* and complete follicle disruption at the highest doses tested (200 and 500 μM). While the initial rise in follicle number was unexpected, the large drop in follicle number was considered a likely outcome, as the exposure window in this study correlated with the time-frame at which both Topo II paralogues (α and β) are expressed within the germ cells. It may well also have been the case that if the *in vivo* study had been carried out at higher doses, then the same drop might have been observed (Fig. 7.1). This therefore suggested that at lower concentrations, there might have been an effect either on germ cell nest breakdown or apoptosis, or both, resulting in the observed initial increase on follicle numbers. The large drop in follicle numbers following high dose exposure *in vitro* however, could be explained by full inhibition of mammalian Topo II, as a result of which most follicles could not undergo meiosis and consequently underwent atresia. Surprisingly however, when the nuclei of exposed cultured oocytes were examined by meiotic spreads, there were some oocytes that were capable of fully synapsing in the presence of AZTC. This suggests either that AZTC does not, in fact inhibit mammalian Topo II, or that Topo II is not required for this early stage of meiosis.

When the post-natal ovary was cultured in the presence of AZTC, the results were even more conflicting (Figure 7.3), since at this stage Topo IIα is only expressed within the somatic cells, whereas Topo IIβ expression is still found within the germ cells. AZTC appeared to target mainly primordial follicles, where the oocyte was the main target of AZTC. Again, this was surprising since the granulosa cells were expected to become a target for AZTC if it was, indeed, inhibiting mammalian Topo

II. It may, therefore, be that AZTC specifically inhibits Topo IIβ. Overall, it is clear, that AZTC does elicit an effect on the ovary, but the exact pathway by which it does so cannot be concluded at this point.

For this reason, another drug that is known to target mammalian Topo II was selected to be used as a comparison alongside AZTC: etoposide (Chen et al., 1984, Ross et al., 1984, Gupta et al., 1987, Fortune and Osheroff, 2000). Furthermore, etoposide was thought to be an interesting and relevant drug of choice because it had already been prescribed to pregnant women, but its effect on the fertility outcome of the sons and daughters born to these women is not yet known. The embryonic ovary culture was therefore repeated with etoposide, and the ovaries were examined histologically for follicle distributions and health. Although the lower doses of AZTC and etoposide did not correlate, there was a significant reduction in size of the follicle pool for both compounds, where it was primarily the primordial follicles that were targeted in AZTC ovaries but also transitional follicles in etoposide-treated ovaries (Figure 7.4). Although the opposite effects were observed for the proportion of transitional follicles between compounds, this could also be explained by the large drop in primordial follicle numbers, resulting in 'proportionally' more transitional follicles per ovary, making it appear as though their numbers have increased. Despite the few similarities, the results largely did not appear to correlate, except for the controls vs. high dose ovaries. Furthermore, in terms of follicle health, neither compound appeared to follow a regular dose-response curve. A post-natal culture was also carried out where newborn (CD-1 P0) ovaries were exposed to etoposide *in vitro,* but etoposide had no significant effects on the post-natal cultured ovary, making it difficult to compare with AZTC. This therefore does suggest that AZTC did not mimic the actions of etoposide, which, in turn, suggests that AZTC may well not block the actions of mammalian Topo II, but further studies would be required to confirm this.

Overall, there are three main possibilities. Either AZTC does directly inhibit mammalian Topo II (on target), or it does not (off-target), or both. If AZTC does indeed inhibit mammalian Topo II, then there are four further possibilities within

this. Firstly, AZTC might inhibit both or either of the Topo II paralogues, where Topo IIα is expressed only within the germ cells pre-natally, and Topo IIβ is expressed within the germ cells throughout life. The second possibility, is that AZTC also inhibits other types of topoisomerases whose expression and function within the mouse ovary has yet to be determined. The third possibility, is that AZTC does inhibit Topo II but that Topo II is not required for this early stage of meiosis, resulting in the observed few nuclei that were able to progress through to the pachytene stage. The fourth and final possibility, is that by inhibiting Topo II, AZTC results in a secondary effect on other cell types. For example, it could act on Topo II within the granulosa cells in the post-natal cultures, which then might affect the signalling loop between granulosa cells and oocytes, consequently resulting in an effect on the oocytes.

If, on the other hand, AZTC does not inhibit mammalian Topo II, it might instead act by a completely different, off-target pathway. If this is the case, as outlined before (Section 5.5.5), AZTC might inhibit Hsp90, a protein involved in cell cycle regulation, cellular homeostasis and steroid hormone signalling, that bears similar topology to the mammalian Topo II molecule (Dunbrack et al., 1997, Prodromou et al., 1997). This would be a particular pathway of interest since an antitumour drug that inhibits Hsp90 has previously been shown to also inhibit mammalian Topo II (Gadelle et al., 2006). If this is the case, it could account for many of the effects observed on the ovary, such as altered E2 levels or cellular homeostasis or an effect on regulation of the cell cycle. E2 protects oocytes from programmed cell death and has been suggested to play a role in follicle formation (Billig et al., 1993, Britt et al., 2004). An alteration in E2 levels in the developing ovary would therefore likely result in altered germ cell nest breakdown (Billig et al., 1993), potentially explaining the observed initial increase in the observed number of primordial follicles. Some compounds, including chemotherapy agents have previously been shown to affect E2 production in the cultured ovary (Oktem and Oktay, 2007), which consequently can affect the primordial follicle pool. Due to the role of E2 in preventing oocytes and granulosa cells from undergoing programmed cell death, disruption of E2 production could induce premature follicle growth and over time, the consequent depletion of

the primordial follicle pool. It would be interesting to investigate if, in fact, the reduction in follicle numbers observed here, in particular, the number of primordial follicles could potentially be a result of altered E2 production in the post-natal ovary.

Figure 7.3. Comparisons between ovaries exposed pre- vs. post-natally (PND4) to AZTC (Sections 5.4.2 & 6.4.2, (or Appendix D) respectively).

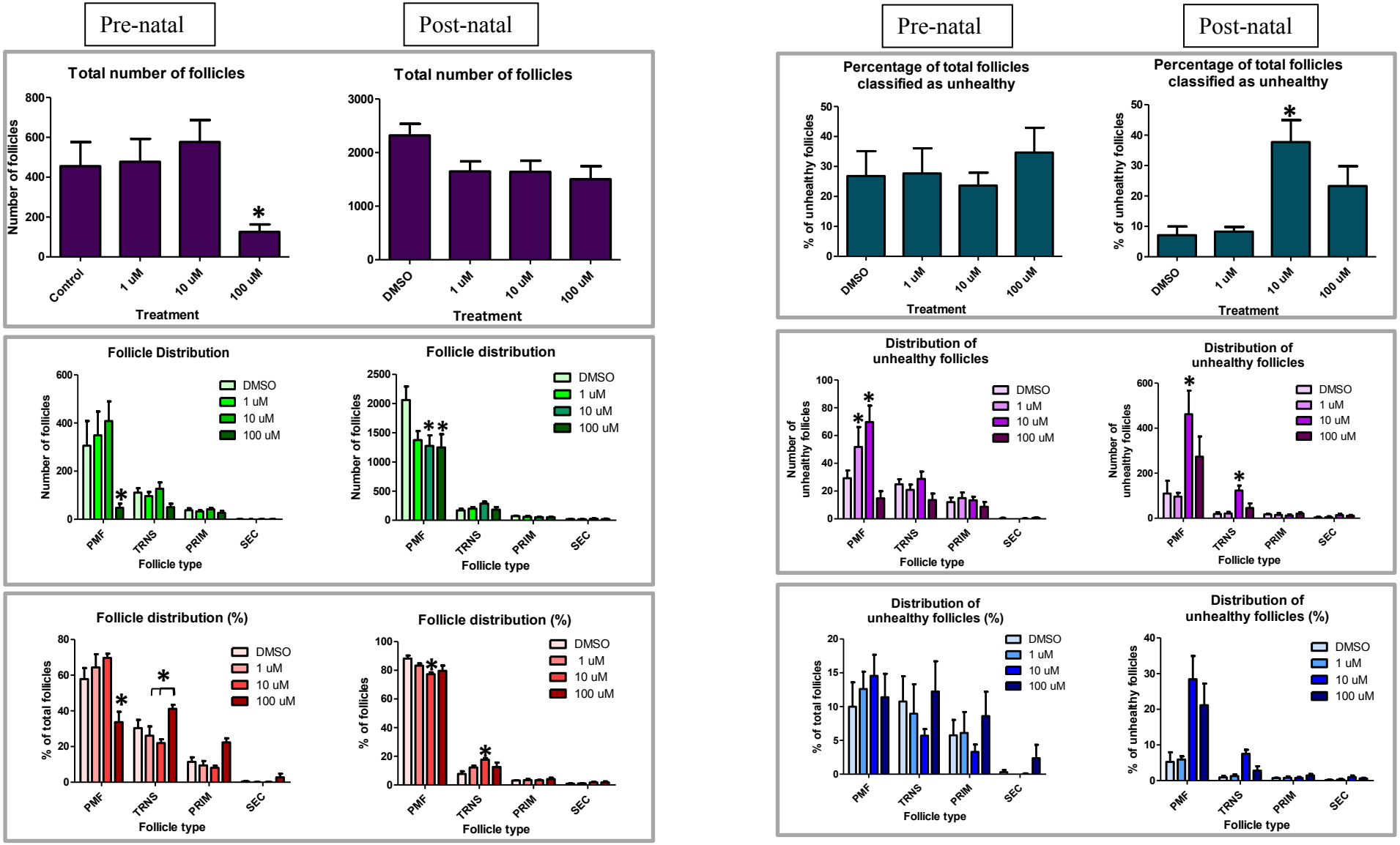
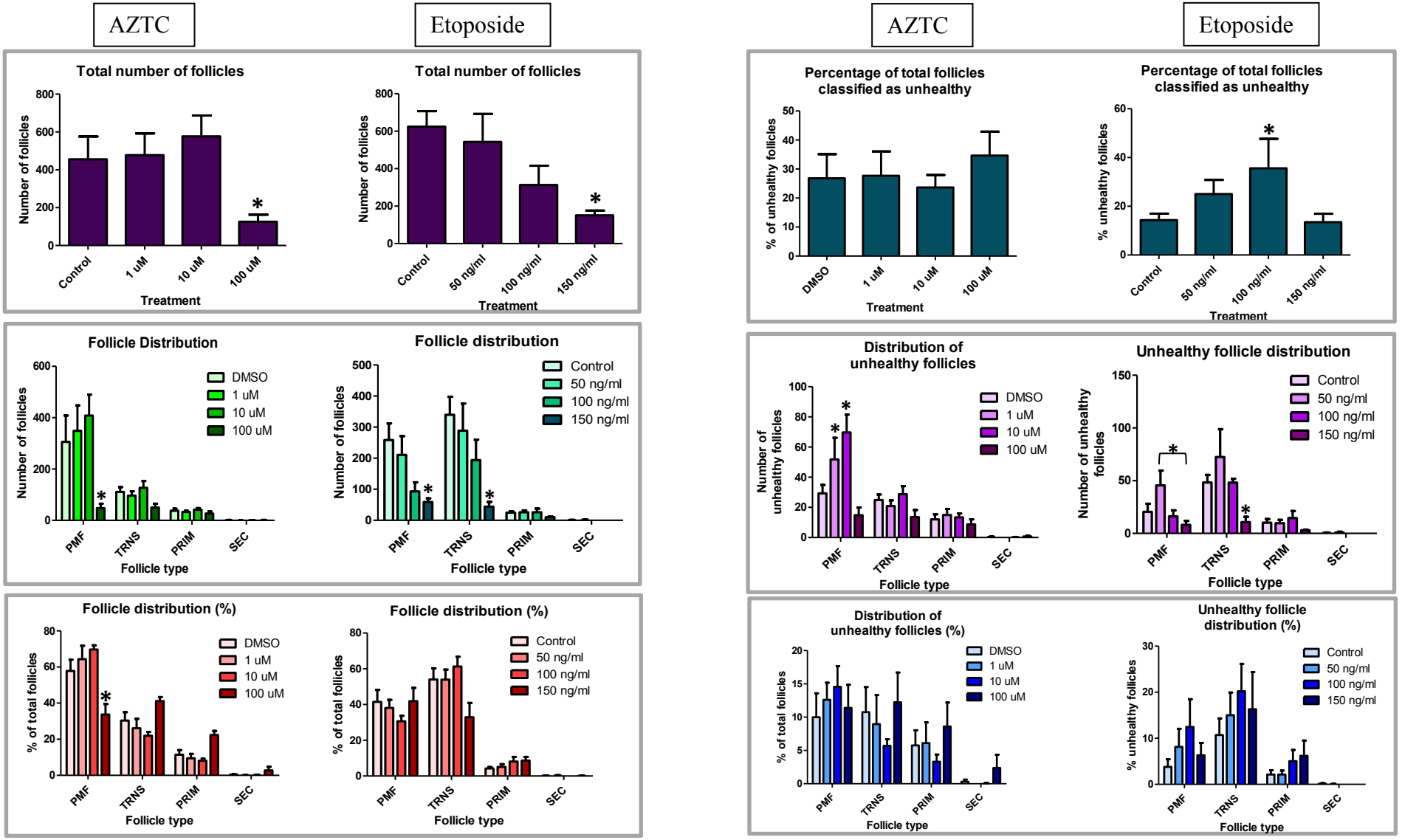


Figure 7.4. Comparisons between ovaries exposed pre-natally to AZTC or etoposide (Sections 5.4.2 and 5.4.3, respectively).



7.6. Future Work

In the future, I think that the main priority should be to expand on the embryonic ovary culture system. The work presented here showed promising results where this culture system could be a useful tool for reproductive toxicology testing. It would be interesting to try carrying out longer cultures, since at the end of the 12 day culture period the ovaries still appeared very healthy, some consisting of a relatively large number of follicles. If some secondary and even pre-antral follicles were to begin to form, it could then be disaggregated and individual follicles could potentially be cultured to the antral stage, to be ovulated and fertilised *in vitro*. If successful, then this would mean that the culture system could cover the entire meiotic division in the female. It would also be interesting to test and assess the efficacy of the culture system further by using compounds with known effects on early meiosis or the pre-natal ovary, such as B[a]P or DMBA for example (Mattison and Thorgeirsson, 1979, Borman et al., 2000, Matikainen et al., 2002, Igawa et al., 2009). Ultimately, the culture system could be attempted using human embryonic ovary tissue; if successful, this could be a very important tool to determine potential ovarian toxicants.

Since the mechanism of action of AZTC was not elucidated during the course of the project, the next step would be to carry this investigation further. Western blot analysis on exposed cultured ovaries could demonstrate whether Topo II levels decreased in the presence of AZTC, and carrying out an immunostain for γH2AX on AZTC-treated ovaries could also demonstrate if AZTC results in a similar increase in DSBs as was observed with etoposide. Furthermore, another experiment that could be carried out, is to do estradiol assays on the culture medium following culture with AZTC, to examine if there is in fact a difference in E2 levels following AZTC exposure. Finally, it would be interesting to investigate whether AZTC does have an effect on meiosis or not, by repeating the chromosome spreads to produce a higher yield of oocytes, or by carrying out immunohistochemistry for diplotene markers on exposed ovaries. Ideally, the *in vivo* study could then be repeated but with AZTC-exposure occurring post-natally, to investigate how our post-natal ovary cultures compare with the *in vivo* situation.

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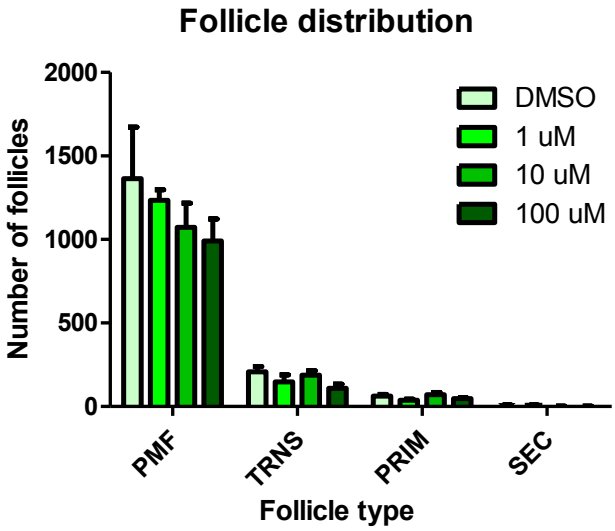
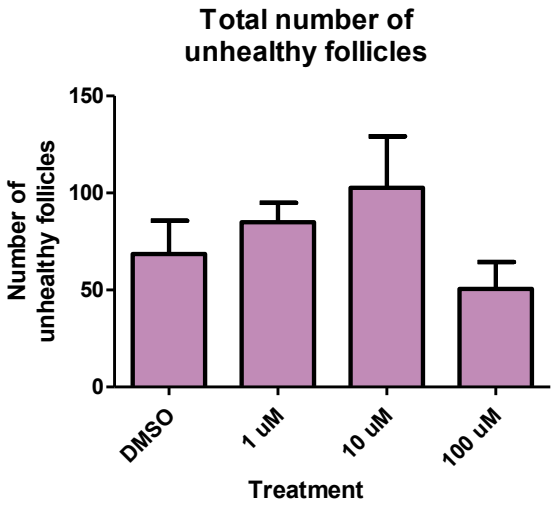
Appendix A. Publication

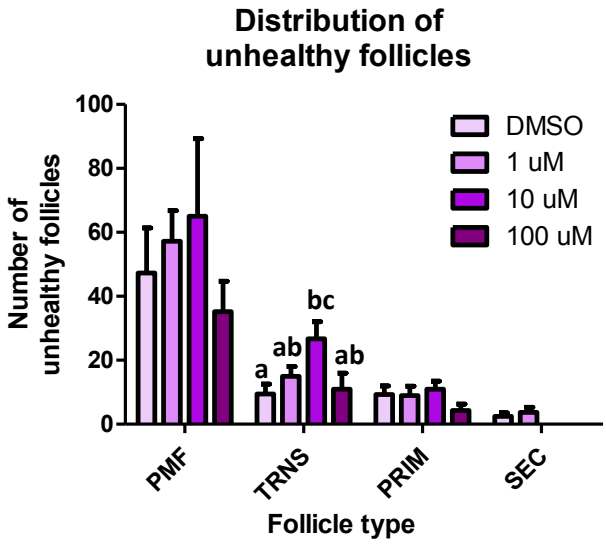
Appendix B. Table outlining the AZTC *in vivo* study design and dose groups.

Group #	Study design	Animal #	Dose of AZTC
1	Ovaries collected on day 15 pp	1,2,5,6	Control
	Ovaries collected at 13+ weeks	343,344, 346, 347, 349, 350	Control
2	Ovaries collected on day 15 pp	8,9,10	Low dose
	Ovaries collected at 13+ weeks	361,357, 365,366, 371,375	Low dose
3	Ovaries collected on day 15 pp	14,16,17	High dose
	Ovaries collected at 13+ weeks	380, 384,385, 392	High dose
4	Ovaries collected at 12 dpc	-	Control
	Ovaries collected at 17 dpc	-	Control
	Ovaries collected at 21 dpc	-	Control
	Ovaries collected on day 0 pp	-	Control
	Ovaries collected on day 5 pp	22,23,28	Control
5	Ovaries collected at 12 dpc	-	High dose
	Ovaries collected at 17 dpc	-	High dose
	Ovaries collected at 21 dpc	-	High dose
	Ovaries collected on day 0 pp	-	High dose
	Ovaries collected on day 5 pp	45,48	High dose

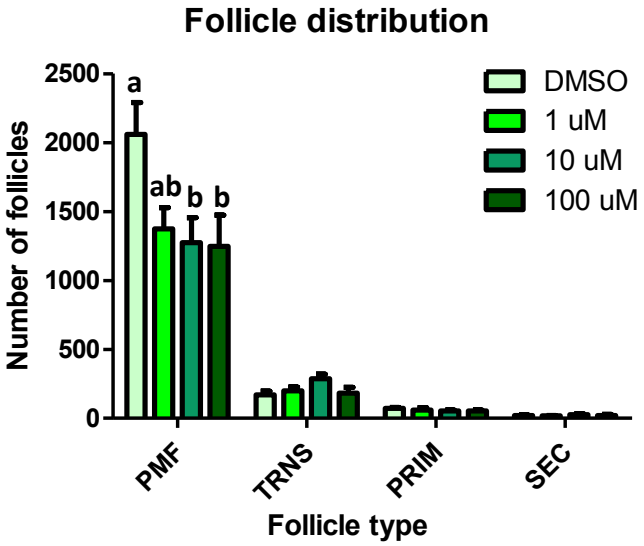
Appendix C. Effect of AZTC on the newborn (P0) CD-1 ovary *in vitro*

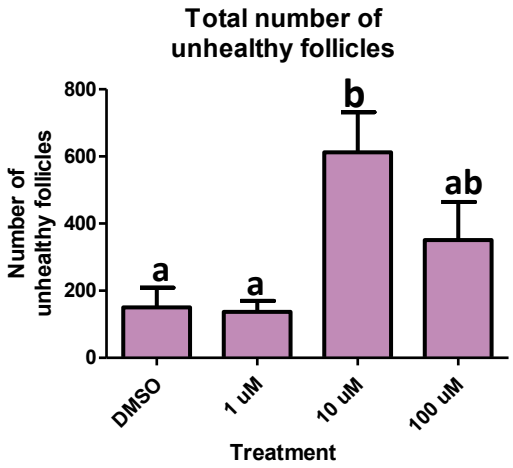


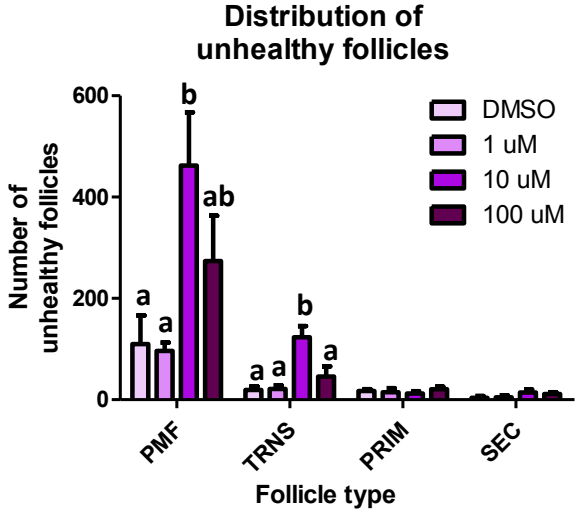




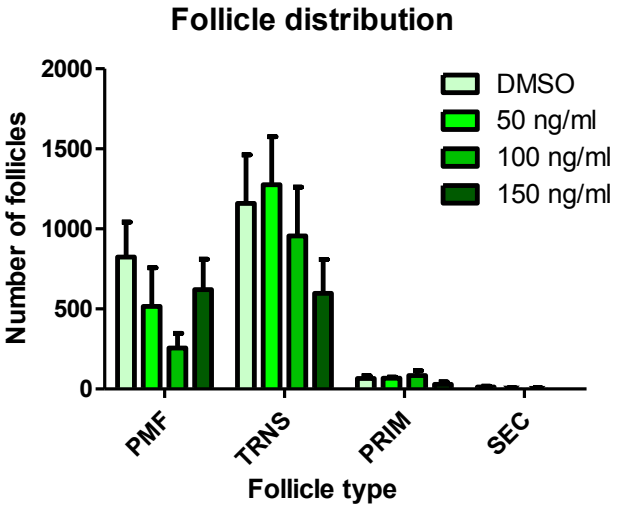
Appendix D. Effects of AZTC on the neonatal (P4) CD-1ovary *in vitro*

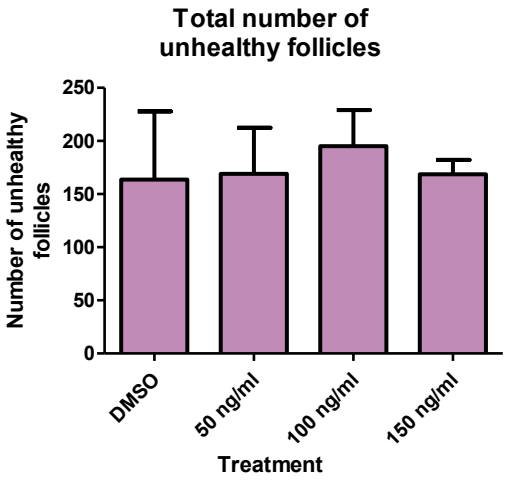


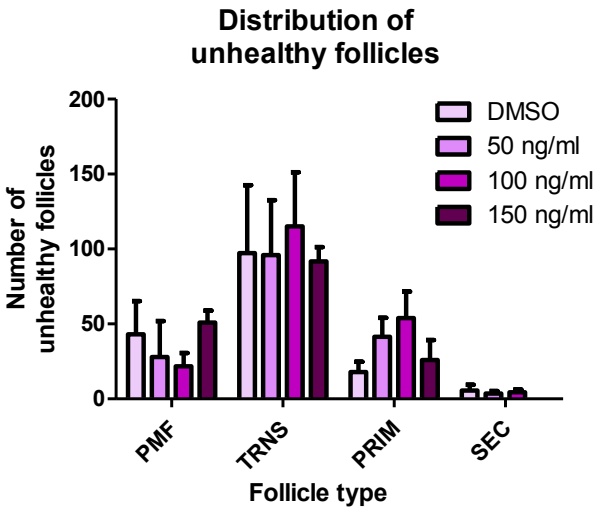




Appendix E. Effects of etoposide on the neonatal (P0) CD-1 ovary *in vitro*







Appendix F. Effect of AZTC on newborn (P0) F1 ovaries *in vitro.*